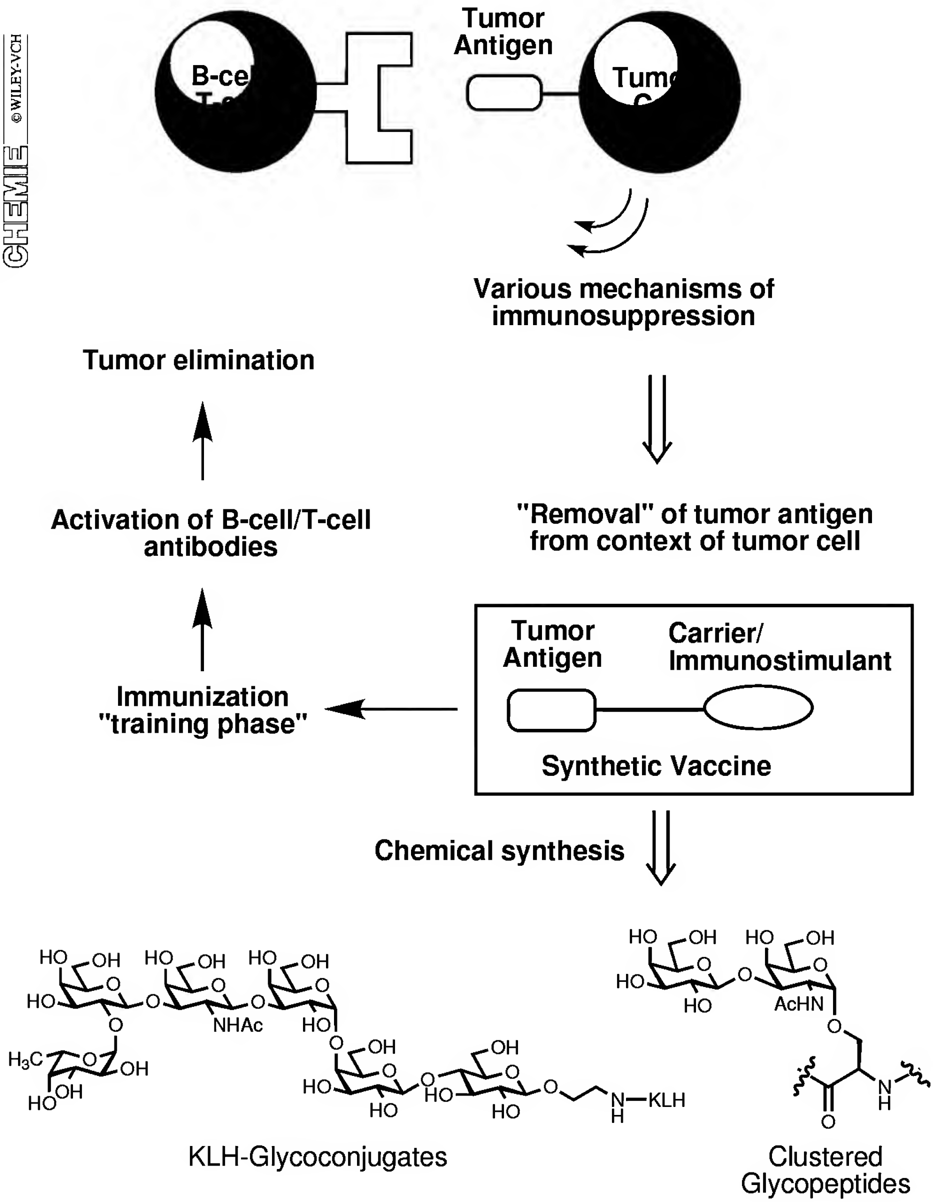
Exhibit A

Development of Carbohydrate-Based Anticancer Vaccines



From the Laboratory to the Clinic: A Retrospective on Fully Synthetic Carbohydrate-Based Anticancer Vaccines**

Samuel J. Danishefsky* and Jennifer R. Allen

Dedicated to Dr. Paul Marks

This review provides an account of our explorations into oligosaccharide and glycoconjugate construction for the creation and evaluation of vaccines based on carbohydrate-centered tumor antigens. Our starting point was the known tendency of transformed cells to express selective carbohydrate motifs in the form of glycoproteins or glycolipids. Anticancer vaccines derived from carbohydrate-based antigens could be effective targets for immune recognition and attack. Obtaining significant quantities of such structures from natural sources is, however, extremely difficult. With the total synthesis of tumor-associated carbohydrate antigens accomplished, we began to evaluate at the clinical level whether the human immune system can respond to such fully synthetic antigens in a focused and useful way. Toward this goal, we have merged the resources of chemistry and immunology in an attack on the problem. The synthesis and immunoconjugation of various tumor-associated carbohydrate antigens and the results of such constructs in mice vaccinations will be described. For fashioning an effective vaccine, conjugation to a suitable immunogenic carrier was necessary and conjugates of KLH (keyhole limpet cyanin) have consistently demonstrated the relevant immunogenicity. Preclinical and clinical studies with synthetic conjugate carbohydrate vaccines show induction of IgM- and IgG-antibody responses. Another approach to anticancer vaccines involves the use of clustered glycopeptides as targets for immune attack. Initial attention has been directed to mucin related O-linked glycopeptides. Synthetic trimeric clusters of glycoepitopes derived from the Tn-, TF- and Lewis^y-antigens, appropriately bioconjugated, have been demonstrated to be immunogenic. The hope is that patients immunized in an adjuvant manner with synthetic carbohydrate vaccines would produce antibodies reactive with cancer cells and that the production of such antibodies would mitigate against tumor spread, thereby enabling a more favorable survival and "quality of life" prognosis.

Keywords: carbohydrates · drug research · glycoconjugates · tumor therapy · vaccines

1. Introduction

The first successful immunization procedure to protect against infectious disease is most commonly credited to Edward Jenner, in recognition of his published findings on the use of cowpox vaccination in 1798 (vaccinia, from the Latin vacca, meaning cow). It was, however, nearly 80 years later that a better comprehension of the mechanisms involved in such phenomena paved the way for the development of modern vaccines by Eulrich, Koch, Metchnikoff, Pasteur, von Behring, and others.[1] A vaccine to induce an anticancer immune response has been a long-standing vision in medicine.[2] However, in contrast to classical viral or bacterial vaccines, which have generally been used to protect from future infections, and conventional cancer therapies, which focus on excision or killing of malignant cells, cancer vaccines are thus far perceived as a mode of treatment subsequent to the detection of the disease. Our emphasis is on the development of vaccine strategies to provide enhanced protection against tumor reoccurrence and metastasis when the tumor burden has been rendered minimal through surgery, radiation, or chemotherapeutic treatment.

Tumor immunotherapy is based on the theory that tumors possess specific antigens that can be recognized when

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[**] Frequently used abbreviations are listed in the appendix.

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presented to or processed by a properly trained immune system. The connection of our laboratory to this field arose from the fact that malignant cells are commonly characterized by the appearance of large and unusual oligosaccharide motifs on their cell surfaces which distinguish them from their normal cell counterparts.[3] The developmental reasons for formation of these oligosaccharide patterns are not completely understood, but the end result is the expression of distinct, cell-surface bound glycolipid or glycoprotein conjugates.[4] Moreover, advances in monoclonal antibody (mAb) technology, immunohistology, and synthetic and structural chemistry have allowed the characterization of a number of carbohydrate-based tumor-associated antigens, providing a series of interesting structures.[5] The exploitation of tumor-associated carbohydrate ensembles to trigger active immunotherapy against cancer cells which express counterpart structures on their surfaces has since been the focus of much investigation, including that in our laboratory. In fact carbohydrate antigens, when administered as a coherent vaccine, have proven to be targets for immune recognition and immune attack.[6, 7]

2. Immunological Considerations

Tumor-bearing hosts fail to reject malignant cells, even in experimentally induced animal tumor models in which the cells express detectable levels of immunogenicity.[8] A conceptual objection can be raised against attempting immunotherapy in humans by using cancer-associated antigens: How can antigens induce an effective immune response when the same antigens expressed by the cancer cells have not done so? Apparently, there exist mechanisms that allow tumor-presented antigens to be seen as "self" by the immune system. These mechanisms, which facilitate tumor growth, have been the subject of much discussion and experimentation.[9] Numerous reports have identified suppressive mechanisms during the growth of immunogenic tumors which serve to provide a rationale for the paradoxical growth of tumors in immunocompetent hosts.[10] In addition, it has been documented that some tumors lack, or shed, crucial recognition molecules which then provides a selective advantage for tumor cells.[11] The goal in the development of anticancer vaccines is to break the tolerance which the immune system has for antigens expressed mainly or exclusively by the tumor. Accordingly, the idea of using synthetically derived cell-free glycoconjugates as versions of immunostimulatory antigens in the development of antitumor vaccines merits a sustained investigation.

The development of increasingly effective vaccines requires follow-up assays to evaluate relevant immunogenicity. These feedbacks serve to guide the process of vaccine construction and testing. With the development of serological typing systems for defining cell-surface antigens, immunologists now

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Under the tutelage of his father, Samuel J. Danishefsky was exposed, at an early age, to the elements of logical thought and critical analysis through the study of the Talmud. He received a B.S. degree at Yeshiva University (1956). In keeping with the example of an older brother (Isadore), he took an interest in chemistry in college. A life long fascination with organic chemistry followed from absorption with two introductory treatments of the subject—one by Raymond Brewster and the other by Louis and Mary Fieser. This exposure led him to pursue graduate studies at Harvard University where he received a Ph.D. (1962) under the direction of Professor Peter Yates. From 1961–1963 he was an NIH sponsored Postdoctoral Fellow at Columbia University under the mentorship of Gilbert Stork. His first independent academic position, which started in 1963, was at the University of Pittsburgh where he became Professor in 1971 and University Professor in 1979. In 1980 he moved to Yale University and served as chairman of the department from 1981–1987. He was named Eugene Higgins Professor in 1984 and Sterling Professor in 1990. In 1993 he returned to New York as Professor of Chemistry at Columbia University and as Kettering Professor and first Head of the Laboratory for Bioorganic Chemistry at the Memorial Sloan–Kettering Cancer Center. His research interests have been in the areas of synthetic strategy, cytoxic natural products, and, most recently, fully synthetic carbohydrate based tumor antigens. In 1996 he shared the Wolf Prize in Chemistry with Gilbert Stork.

Jennifer Allen was born as Jennifer Lowe in Cleveland, Ohio. She received her A.B. degree in Chemistry in 1992 from Miami University in Oxford, Ohio, and her Ph.D. from Duke University in 1998. Her doctoral research, under the supervision of Ned A. Porter, examined the synthesis and applications of unsymmetrically labeled alkyl hydroperoxides. Particular emphasis was on mechanistic studies of the [2,3]-allylperoxyl rearrangement. As a postdoctoral fellow with Professor Danishefsky she is attempting to fashion new anticancer vaccines via fully synthetic carbohydrate-based tumor antigens. Her areas of interest include glycosidic rearrangements for the construction of glycosides, glycoconjugates, and glycomimetics and studies which allow for a better understanding of the biological phenomena in which carbohydrates participate.

have assays of requisite sensitivity and specificity which are comparable to those used in the monitoring of vaccines against infectious diseases.[12] Furthermore, effective assays capable of detecting T-lymphocyte responses, while still lagging behind, may soon provide an opportunity for evaluation of the progress with T-cell responsive antigens. Thus, immunogenicity to cancer vaccines can be established through the use of well-defined tumor antigens, whose structure and quantitative expression levels on tumors and normal cells in vivo are known.

There are grounds for both optimism and caution in using cancer vaccines which contain carbohydrate antigens as major stimulants. Carbohydrate-based vaccines have thus far been unsuccessful in inducing detectable T-cell immunity. As a result, sole reliance on carbohydrate antigens for immunotherapy of cancer can be limiting.

In general, immune responses against carbohydrate antigens are largely restricted to inducement of antibodies. Antibodies used in this way could provide a mechanism for eradication of circulating tumor cells (in the blood stream) and micrometastases, thus providing protection from tumor reoccurrence. The mechanism of protection could involve attack and lysis, mediated by other blood plasma substances, with the cell-surface carbohydrate antigens as targets; however, there may be other operative mechanisms. Experimentation involving administration of monoclonal antibodies against carbohydrate antigens supports this mechanism and treatment in mouse models has progressed to the point where micrometastases can apparently be eliminated.[13] Cancer patients in clinical studies have also responded favorably to natural or passively administered antibodies, resulting in prolonged disease-free survival and prognosis.[14] With repeated occurrences of metastases controllably minimized as a consequence of high levels of circulating antibodies, aggressive local therapies followed by vaccination could result in long-term control over even metastatic cancers. It is clear that there is potential for immunotherapy of cancers using monoclonal antibodies.[15]

There is another potentially complicating feature in the use of carbohydrate-based tumor antigens in cancer vaccines. Cancer and normal cells growing in tissue culture generally show minimal levels of expression of such antigens. The immense difficulties associated with their purification from such sources render them virtually nonavailable as homogeneous starting materials for a clinical program. Accordingly, it falls to organic chemists to play a key role in the development of such cancer vaccines. The first role of the synthetic chemist is that of solving purity and availability problems if the program is to have any chance of advancing to a clinical setting. Moreover, chemistry must play a major role in the conjugation phase, which is decisive in upgrading a synthetic antigen to a vaccine. Although they do show minimal activity, most tumor antigens, including carbohydrate tumor antigens, are generally poor immunogens. Rather they require an appropriate immunogenic carrier to achieve an optimal response.[16] Therefore, an additional challenge to cancer vaccine strategies is the successful delivery of the synthetic tumor antigens in a favorable molecular context for eliciting a therapeutically useful immunological response. Collectively, there is an ample basis for studying carbohydrate antigen-based vaccines to promote the production of antibodies in a clinical setting.

Our laboratory has been engaged in synthesizing complex oligosaccharides and glycoconjugates for some time. In addition to their aforementioned role as tumor antigens, carbohydrates can influence many biological events. They mediate a variety of functions including inflammation, control of growth and differentiation, cell–cell adhesion,[17] as well as being determinants in blood group typing.[18] Given these facts and given our synthetic advances in the preparation of complex carbohydrates, the next logical step in our program was to explore the total synthesis of carbohydrate-based tumor antigens as part of a coherent vaccinology program. From our viewpoint, emphasis was placed on the assembly of carbohydrate-based vaccine constructs that would otherwise be unavailable from natural sources or from other isolation methods.

At the Sloan–Kettering Institute, our laboratory is in active collaboration with the Laboratory for Tumor Vaccinology and the Laboratory for Tumor Antigen Immunochemistry and is closely associated with various clinical facilities in the Hospital of the Memorial Sloan–Kettering Cancer Center (MSKCC). This review will report on our continuing chemical studies into the development of synthetic methodology of general applicability for the preparation of carbohydrates, in the form of both glycolipids and glycopeptides, which mimic components of the cell surface of tumor cells. This progress has allowed us to assemble a number of vaccine constructs containing tumor-associated antigens which have been clinically evaluated and are in ongoing human trials (Figure 1). We emphasize that this account is not meant to be a formal and exhaustive treatment of the whole subject. Rather it should be viewed as a retrospective on our own activities, stressing not only the chemistry which lay at the heart of the effort, but also the way in which the chemistry was interfaced with allied disciplines to move the program forward. There are certainly others also currently working in the same direction using synthetic conjugates and we point the reader to accounts of their important work.[19, 96, 97]

We describe here the synthesis and immunological evaluation of neoglycoconjugates which contain the following antigens: the MBr1 antigen Globo-H (**1**), the adenocarcinoma antigen KH-1 (**2**), the blood group determinant and ovarian cancer antigen Lewis^y (**3**), the major and minor N3 antigens associated with gastrointestinal cancer (**4a** and **4b**), and the small cell lung carcinoma antigen fucosyl GM_1 (**7**). In addition, we have investigated clustered mucin-related structures of O-linked antigens containing Lewis^y, the Tn (**5**) and TF (**6**) antigens. All of the vaccine constructs reported here have been synthesized in appropriate bioconjugatable form and been through proper mouse immunization studies. This has lead to a "proof of principle" clinical evaluation of three of our fully synthetically derived vaccines (Globo-H in prostate and breast cancer trials, Lewis^y–KLH conjugate in ovarian cancer trials and Tn/TF clustered, O-linked mucin models in prostate cancer trials. The Globo-H–KLH conjugate is poised to enter phase II and phase III human clinical trials). The case histories of these constructs will be described

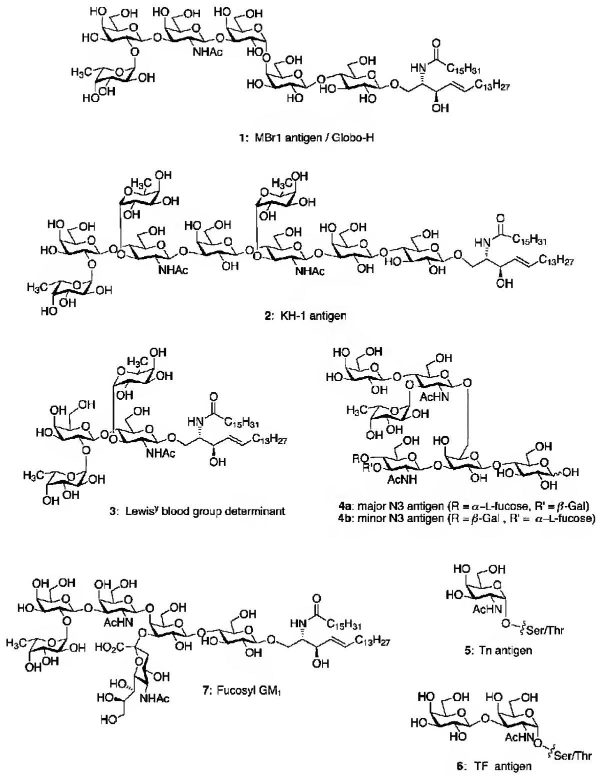


Figure 1. Structures of tumor associated antigens described in this account.

with emphasis on the rationale followed for progression from the drawing board through the lab and on to immunocharacterization en route to mouse vaccinations which terminate, in favorable cases, with clinical trials. In terms of the final structures, these represent the most complex, fully synthetically derived constructs ever to be clinically evaluated.

3. Synthetic Considerations

Synthesis is first and foremost an eminently practiced subject with primary emphasis on issues of conciseness and efficiency. Thus the best syntheses are the ones which address these goals most successfully. However, in pursuing this program we also sought a unifying framework around which to organize our thinking. In this connection we turned to the glycal assembly methods which have been developed over many years in our laboratory. The logic and details of glycal assembly have been amply reviewed.[20] In this document we do not review the matter further except by illustration.

Several issues merit particular consideration when contemplating the synthesis of complex glycoconjugate-containing substructures, such as those in Figure 1. From the standpoint of synthetic economy, it is beneficial to gain significant relief from the protecting group manipulations which have come to dominate traditional syntheses of complex branched oligosaccharides. It is in this area that we perceived a great advantage in using glycal building blocks to rapidly buildup the oligosaccharides of carbohydrate-based tumor antigens.

Another determining issue for the vaccine project involved establishing a linker domain through a spacer unit, for purpose of creating a functional immunogen. Attachment of an appropriate immunostimulant to the carbohydrate would follow. Although the optimal spacer–linker combinations are not well-established to date, the overall goal is that the molecular recognition of the synthetic tumor antigen by the immune system is not compromised by the conjugation of the carbohydrate domain to an effective biocarrier.[21] In this context, we began with an exploration to fashion the carbohydrate antigen attached to an appropriate carrier protein (keyhole limpet hemocyanin, KLH). The conjugation strategy which we elected relies on the protocol of Bernstein and Hall,[22] which calls for reductive coupling of a glycoside, which terminates in a glycoaldehyde, with the intended protein carrier, presumably at the ε-amino acid residues of exposed lysines. In a second-generation synthetic plan, we envisioned an option for clustering of the antigens via suitable peptide couplings to generate mucin-like structures.[23] A general method for solving the stereochemical issues associated with the construction of α-serine/threonine O-linked oligosaccharides was developed with the help of a "cassette" approach (see Section 9). With the glycopeptides, we also explored different immunostimulatory moieties in order to circumvent the requirement for conjugation of the complex construct to a carrier protein.

In summary, the preclinical goals at the outset of the vaccine program, with respect to the various tumor antigens investigated, were the following:

a) Total synthesis of the desired tumor-associated antigen, chemically rigorous proof of structure as well as homogeneity, and, when appropriate, synthesis of relevant truncated congeners as probes of epitope specificity.
b) Incorporation of an appropriate spacer group so that the immunological integrity of the antigens is retained.
c) Covalent contact with an immunostimulant or carrier protein to generate a fully functional vaccine.
d) Mouse immunization and follow-up evaluation of immune response.

Petition for human clinical trials would have to await the attainment of these goals. The interface of the glycal assembly logic with these strategies and goals is depicted in the paradigm shown in Figure 2.

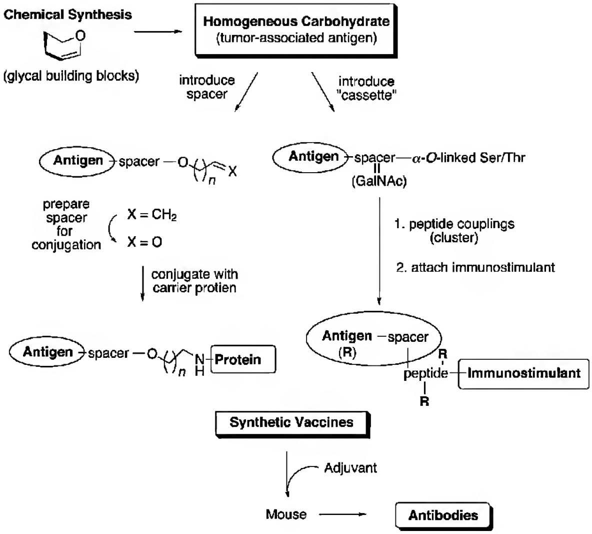


Figure 2. General approach to synthetic carbohydrate vaccines.

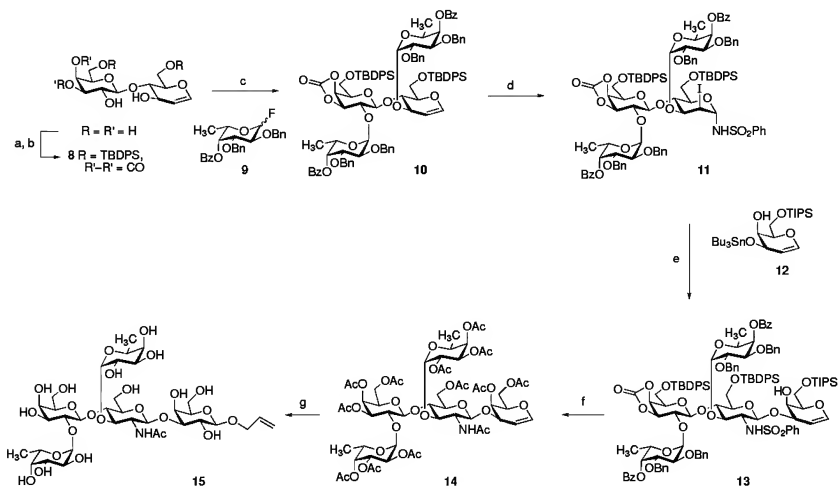
4. The Lewis^y – KLH Glycoconjugate

4.1. Synthesis of the Le^y – KLH Glycoconjugate

In 1995 our synthesis of neoglycoconjugates of Lewis^y (Le^y) and Lewis^b (Le^b) blood group determinants and of H-Type I and H-Type II oligosaccharides was described.[24] The Le^y determinant was of particular interest to us because it had been previously identified as an important epitope for eliciting antibodies against colon and liver carcinomas.[25] It has also recently been implicated as a marker in metastatic prostate cancer and was found to be overexpressed in ovarian tumors.[26]

In keeping with the objectives outlined above, the initial goal was the synthesis of a bioconjugatable precursor of the Le^y epitope **3** (Figure 1) for incorporation into a coherent vaccine. The successful fashioning and implementation of the synthesis, organized around Le^y, was a critical development en route to future vaccine constructs. In addition, it serves as a guiding paradigm for the use of glycal assembly methods and logic.

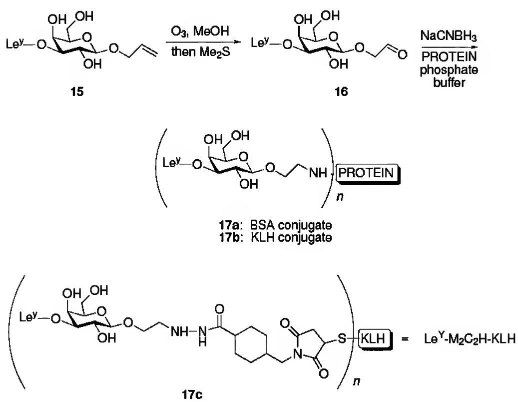
The pentasaccharide containing the Le^y specificity was prepared as shown in Scheme 1. The route took full advantage of the *N*-acetyllactosamine backbone in the target. Readily available lactal[27] was silylated at the two primary sites. This selective protection phase was followed by cyclic carbonate formation through the *cis*-C3′,C4′ sites to give **8**. The resulting exposure of the C3 and C2′ hydroxyls was of particular significance in this synthesis. Thus, the stage was now set for the bis-fucosylation of **8** to provide access to the Le^y-series tetrasaccharide. In the event, bis-fucosylation[28] of acceptor **8** was carried out with fluorinated donor **9**,[29] thereby providing the Le^y tetrasaccharide as a glycal, **10**. The nonparticipatory benzyl ether at C2 and a potentially participating benzoate at C4 of donor **9** had conspired to provide the required α-selectivity. Next, the glycal functionality was readied to act as an azaglycosylation



Scheme 1. Synthesis of the Lewis^y allyl glycoside. a) TBDPSCl, imidazole, DMF, 84 %; b) Carbonyl diimidazole, THF, 58 %; c) $AgClO_4$, $SnCl_2$, DTBP, Et_2O, 51 %; d) $PhSO_2NH_2$, $I(coll)_2ClO_4$, 99 %; e) $AgBF_4$, THF, 75 %; f) 1. TBAF, THF; 2. Na/NH_3, MeOH; 3. Ac_2O, pyridine, 37 %; g) 1. DMDO, CH_2Cl_2; 2. allyl alcohol, $ZnCl_2$, THF; 3. NaOMe, MeOH, 72 %; 3 steps.

donor[30] by using our previously developed iodosulfonamidation protocol. Use of the iodosulfonamide **11** to glycosylate the galactal tin ether **12** was subsequently achieved with silver tetrafluoroborate to give the pentasaccharide glycal **13**. The desired amino functionality and β-selectivity were installed in the rollover with the galactal acceptor. Global deprotection followed by peracetylation afforded the derivative **14**. This compound later proved to be an extremely valuable intermediate, quite aside from its role en route to **15** (see O-linked system, vide infra).

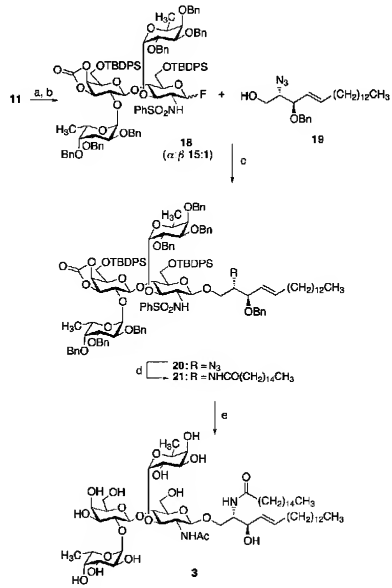
With the glycal **14** in hand, the necessary chemistry for conjugation to the carrier protein was implemented. Treatment of **14** with dimethyldioxirane,[31] followed by opening of the resulting epoxide with allyl alcohol selectively gave the desired allyl glycoside. Removal of the ester protecting groups with catalytic sodium methoxide gave the fully deprotected Le^y pentasaccharide **15** ready for the conjugation phase. In the event, **15** was ozonolyzed in MeOH at -78°C and subjected to a workup with dimethyl sulfide to yield the uncharacterized aldehyde intermediate **16**, as shown in Scheme 2.



Scheme 2. Conjugation of allyl glycoside **15**.

As previously mentioned, the reductive amination protocol of Bernstein and Hall[22] was applied to the case at hand with bovine serum albumin (BSA) in phosphate buffer (pH 8.0) to afford the protein conjugate **17a** which was purified by exhaustive dialysis. Analysis[32] following cleavage of the glycosidic linkages with TFA showed the expected sugar composition: 2 parts galactose, 2 parts fucose and 1 part glucosamine. Analyses of the carbohydrate:protein ratio showed the substitution of an average of 15 Le^y moieties per carrier molecule. Subsequent studies of the installation of carrier protein KLH to give **17b** by an analogous reductive amination procedure provided glycoconjugates with an average of 287 Le^y moieties per molecule of KLH.

To complete the glycolipid total synthesis objective, the ceramide-linked glycoconjugate of the Le^y epitope was also prepared. Returning to the iodosulfonamide **11**, conversion into the fluoro donor **18** was accomplished in a two-step procedure, as shown in Scheme 3.[33] Donor **18** (15:1 α:β) was coupled to compound **19**, the well known azidosphingosine precursor,[34] using a mixed metal promotion system involving zirconocene dichloride and silver triflate to yield the Le^y sphingosine **20**. Reduction of the azide followed by installation of the ceramide with palmitic anhydride to the resulting amine was accomplished, albeit in low yield, to give **21**. Other conditions to reduce the azide in this system remain to be explored, although it will be seen that similar conditions with alternative oligosaccharides proved to be quite successful. Global deprotection of **21** as before yielded the known Le^y glycolipid conjugate **3**.



Scheme 3. Synthesis of the Lewis^y sphingoglycolipid. a) THF/H_2O, TEA, Ag_2CO_3; b) DAST, THF; c) [Cp_2ZrCl_2], AgOTf, CH_2Cl_2, 57%; d) H_2/Lindlar's catalyst, palmitic anhydride, 22%; e) 1. TBAF, THF; 2. Na/NH_3; MeOH; 3. Ac_2O, Pyridine; 4. NaOMe, MeOH.

The successful synthesis of the Le^y constructs **17a**, **17b**, and **3** marked the beginnings of our immunological collaborations and set the stage for further investigations into the synthesis of antigenic glycoconjugates. In addition, in conjunction with the described studies, the synthesis and bioconjugation of a Lewis^b hexasaccharide was also completed using analogous methodology.[24] Thus with these synthetic endeavors, it was clear that we had secured sufficient protocols for protein conjugation with carbohydrates prepared by glycal assembly methods (see Scheme 2).

4.2. Mouse Immunizations with Synthetic Le^y–KLH Glycoconjugate

With a view to developing Le^y-based vaccines, we examined the immunogenicity of the Le^y conjugates in mice. The

production of antibodies as a direct result of immunization was of interest. A critical question to be addressed was whether antibodies thus elicited would show binding reactivity with cancer cell lines expressing the Le^y epitope. Determination of lysis to such cancer cells would also be important. Demonstration of positive results would constitute a significant starting point for human trials. Needless to say, a critical control would demonstrate that the induced antibodies were unreactive with Le^y negative cell lines and other controls. The results of the mouse immunization studies that were obtained are summarized below.[35] As in all of our studies, the vaccines were coadministered with the immunological adjuvant QS-21.[36]

In addition to synthetic conjugates **17a** and **17b**, a related synthesis[37] provided a maleido-derivatized KLH conjugate **17c** (see Scheme 2). Immunization of groups of mice with the three conjugates, together with the adjuvant QS-21, showed that the Le^y oligosaccharide linked directly to KLH **17b** was the most efficient for eliciting both IgG and IgM antibody responses to natural forms of Le^y epitopes carried on mucins and glycolipids.[38] As determined by an ELISA (enzyme linked immunosorbant assay), the IgM antibody responses were typically much higher in titer than the IgG responses. The antisera obtained following immunization with **17b** were also tested, using an immune adherence assay, for their reactivity with Le^y expressing tumor cells. It was found that the antisera from the Le^y–KLH immunized mice strongly bound Le^y-positive cell lines (MCF-7) and not Le^y-negative cell lines (SK-MEL-28).

Cytotoxicity tests for antibody-dependent complement-mediated lysis were also carried out and the results are shown in Table 1. Again, immune adherence assays were used to detect complement-binding antibodies (mainly IgM) which were subsequently found, in the presence of human complement, to be cytotoxic to Le^y-positive MCF-7 cell lines. Furthermore, the antibodies detected by this assay were not cytotoxic to SK-MEL-28 cell lines.

In summary, these studies showed that mice immunized with **17b**, together with QS-21, produced high titers of both IgG and IgM antibodies capable of reacting with epitopes carried on tumor cells expressing Le^y. These studies provided the basis of protocols which were drafted to guide a phase I clinical trial. Following approval from various regulatory bodies, resynthesis of the vaccine under conditions and specfications suitable for human trials was necessary. We then moved onto phase I trials using human patients with ovarian cancers. The goal of the phase I trials was to test the safety of the vaccine and any antibodies that may be produced in response to the vaccine. The results of these studies have now been evaluated and show positive serological findings. These clinical results will be presented separately.

Table 1. Cytotoxicity assay of antisera on cultured cells.

Antibody or antiserum	MCF-7[a] (Le^y +)	SK-MEL-28[a] (Le^y −)
mouse 1 (Le^y-KLH)	1:80	< 1:10
mouse 2 (Le^y-KLH)	1:40	< 1:10
mouse 3 (Le^y-M_2C_2H-KLH)	1:40	< 1:10
mouse 4 (Le^y-M_2C_2H-KLH)	1:40	< 1:10
3S193 (anti-Le^y)	0.5 $\mu g\,mL^{-1}$	> 10 $\mu g\,mL^{-1}$
R24 (anti-GD3)	> 10 $\mu g\,mL^{-1}$	0.62 $\mu g\,mL^{-1}$

[a] Greatest dilution giving detectable lysis; no lysis (< 1:10) was observed in pre-immunization sera.

5. The MBr1 Antigen Globo-H

5.1. Synthesis of Glycoconjugates Containing the MBr1 Antigen Globo-H

Globo-H **1** (Figure 1) is a hexasaccharide which was isolated in submilligram quantities as a ceramide-linked glycolipid from the human breast cancer cell line MCF-7 by Hakomori et al.[39] It is expressed at the cancer cell surface as a glycolipid, and possibly a glycoprotein. Another advance, which sparked interest in this antigen, was its immunocharacterization via monoclonal antibody (mAb) MBr1 by Colnaghi and co-workers, where the antibody had been obtained from mice immunized with intact MCF-7 cell lines.[40] The isolation of **1** from these cell lines and its binding to MBr1 were therefore taken to implicate this glycolipid as a breast tumor antigen. In addition, Globo-H was more recently immunocharacterized (mAb VK-9) by Lloyd et al.[41] Subsequent immunohistological analysis with MBr1 found that the antigen was also expressed in other types of carcinomas including colon, lung, ovary, and small cell lung cancers.[42] Globo-H has also been detected in the majority of the carcinomas of the pancreas, stomach, uterine endometrium, and, in particular, was found be to expressed in both primary and metastatic prostate cancer specimens.

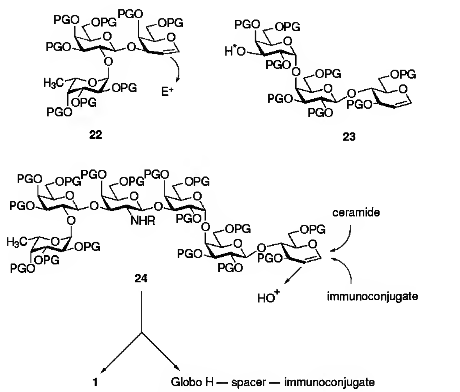
There is also evidence that there are cell surface carbohydrates, assumed to be Globo-H, which react with the MBr1 antibody on normal breast, pancreas, small bowel, and prostate tissues. The antigen in these tissues is, however, predominantly localized where access to the immune system is restricted.[43] Therefore, results using the criterion of immunohistology, which are based on the binding of MBr1 to define the presence of Globo-H as a total structural entity in a particular tissue, should be viewed with caution.

As a result, only experimentation would reveal whether Globo-H is a useful antigen in mouse immunizations and would be of value in cancer treatment in the adjuvant setting. Indeed, at this stage only synthesis could confirm that **1** is the actual antigen recognized by MBr1 and only synthesis could provide sufficient quantities for the immunization studies. Even before we progressed far in the total synthesis, it was recognized that mapping studies with truncated versions of **1**,[44] to investigate which portions of **1** are necessary for binding, would also be of interest to probe these intimate questions in more detail.[45] Collectively, these findings provided the rationale for evaluating Globo-H as a candidate target antigen and rendered it an important synthetic target. At the outset of the Globo-H program, no total syntheses of the antigen had been accomplished.[46]

We now relate the key steps in our total synthesis of constructs corresponding to the carbohydrate sector of **1**. We go on from there to describe the construction of the proposed

vaccine containing the MBr1 antigen. The serological data starting from mouse immunizations and progression through a recently completed phase I trial are reported.

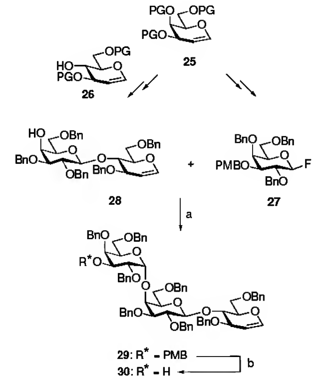
In studying the structure of Globo-H **1** with respect to a venture in chemical synthesis, we were mindful of an important requirement. Our first sub-goal would be, as usual, an academic type solution that might produce adequate quantities (5–10 mg) for proof of structure, immunocharacterization, conjugation, and mouse vaccinations. However, the synthesis eventually had to be capable of producing much larger amounts if the serological findings were positive and if the intent were to move toward clinical trials. We came to favor a disconnection into two trisaccharides, as shown in Scheme 4. In anticipation of a [3+3] coupling to bring the two



Scheme 4. Retrosynthesis of the MBr1 antigen, **1**. E^+ = electrophile (acetamido equivalent), PG = protecting group.

trisaccharides together, glycal **22** emerged eventually to play the role of donor. Glycal **23**, with the appropriate oxygen distinguished at C3 of the galactal residue at the nonreducing end of the trisaccharide, would function as the acceptor. As in the synthesis of Le^y, system **22** would be converted to an azaglycosylation donor upon activation of its glycal linkage. The desired amino functionality would result through glycosylation with **23**. In the forward sense, formation of hexasaccharide **24** now allows for introduction of the ceramide moiety via the glycal linkage to yield the natural form of Globo-H, as well as formation of a suitable immunoconjugate through a linker sector to create a vaccine construct.

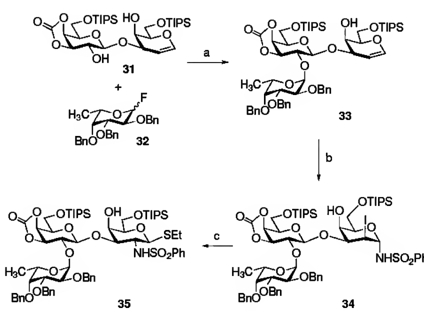
The plan charted in Scheme 4 was implemented[47] and the first total synthesis of **1** was reported in 1996.[48] We begin with the synthesis of the acceptor trisaccharide corresponding to **23** (Scheme 5). Both fluoro donor **27** and lactal acceptor **28** were obtained using galactal **25** and glucal **26** type building blocks. Donor **27** contains the differentially protected PMB ether at C3. In the subsequent [2+1] coupling, acceptor **27** and donor **28** were combined to provide the trisaccharide glycal **29** using a modified version of the conditions described by Mukaiyama



Scheme 5. Synthesis of the trisaccharide acceptor. a) $AgClO_4$, $SnCl_2$, DTBP, Et_2O, 54%; b) DDQ, CH_2Cl_2, H_2O, 86%.

et al.[28] and Nicolaou and co-workers.[49] Oxidative deprotection of the PMB ether afforded **30** which was poised for coupling with the appropriate trisaccharide donor.

Construction of the donor commenced with disaccharide **31** (itself available by two galactal building blocks of type **25**), as shown in Scheme 6. Regioselective fucosylation of the C2 equatorial hydroxyl with donor **32** provided the trisaccharide



Scheme 6. Construction of the trisaccharide donor. a) $AgClO_4$, $SnCl_2$, DTBP, Et_2O, 47%; b) $I(coll)_2ClO_4$, $PhSO_2NH_2$, 4 Å molecular sieves, THF; c) EtSH, LHMDS, DMF, $-40 \rightarrow 0\,°C$; 40% over 2 steps.

glycal **33**. The latter was converted to iodosulfonamide **34** under standard conditions. Even after careful and repeated investigation, it was found that union of acceptor **30** with trisaccharide substrates, such as **34**, could not be accomplished by direct azaglycosylation in a serviceable yield. Although this type of merger with simpler substrates provided a very powerful entry to somewhat simpler constructs (see Le^y), the method proved to be unreliable with severely hindered acceptors.

Fortunately, a solution did present itself. We had earlier explored the conversion of iodosulfonamides into more generally competent donors which might be effective in

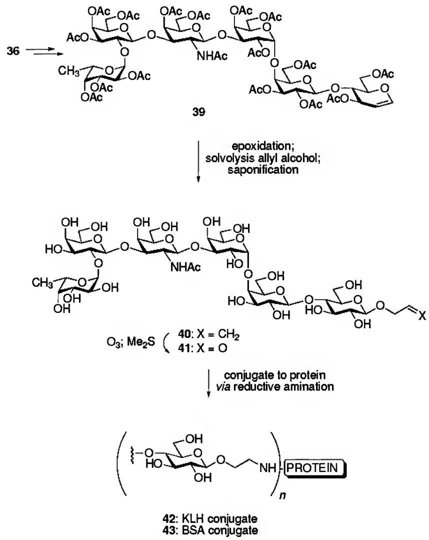
otherwise difficult cases.[30] These findings were applied to the case at hand, in the hope that a successful coupling would result in the formation of the β-configured product via sulfonamide participation. Following this lead, iodosulfonamide **34** was treated with lithium ethanethiolate to afford the requisite thioethyl donor **35**.

The cornerstone of the synthesis is depicted in Scheme 7. The critical coupling of **30** and **35** was effected by the action of stoichiometric MeOTf as promoter of the thioethyl donor, to give hexasaccharide glycal **36**. This was a splendid demonstration that the two-stage formation of *trans*-2-sulfonamido-β-thioglycosides from glycals could be very useful for the coupling of complex fragments when direct glycosylation employing the corresponding iodosulfonamide fails.

To yield the naturally occurring globoside, the properly configured hexasaccharide was epoxidized, treated with sphingosine precursor **19**,[34] and acetylated (Scheme 7) to yield **37**. Reduction of the azide using H_2/Lindlar's catalyst in the presence of palmitic anhydride provided ceramide **38**, in excellent yield. Global deprotection using standard procedures, followed by acetylation of the crude mixture, and saponification yielded glycosphingolipid **1**, whose spectral properties at the various anomeric centers were in complete agreement with those published for the natural material. A detailed proton NMR spectrum of the purified MBr1 antigen from tissue collection had not previously been available due to lack of material. However, our ^1H and ^{13}C spectra as well as mass spectral measurements, in conjunction with the spectra which reflected the synthetic progression, provided convincing support as to the chemical structure of the final product.

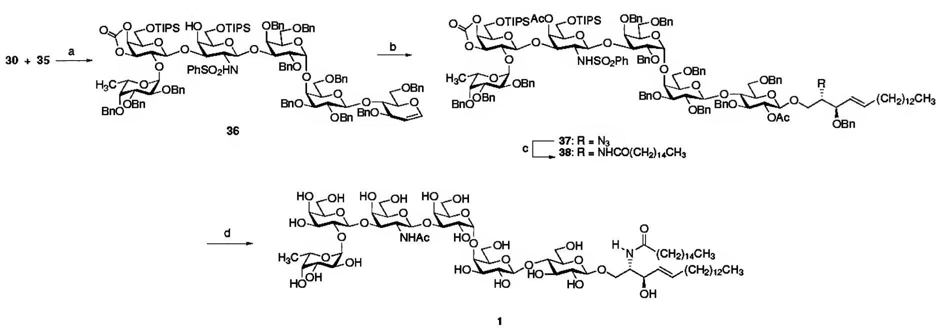
At the immunocharacterization level, synthetic compound **1** was shown by ELISA and immune thin layer chromatography assays to bind to monoclonal antibody MBr1. Inhibition studies revealed that preincubation of MBr1 with **1** completely inhibits MBr1 reactivity with human breast cancer cell line MCF-7. Thus, synthetic glycosphingolipid **1** contains the same antigenic epitope with which MBr1 reacts on breast cancer cells.

We next turned our attention to fashioning a functional vaccine which contained the synthetic antigen. In accordance with our previous studies, we constructed the corresponding allyl glycoside to allow for carrier protein conjugation (Scheme 8). For this purpose we returned to hexasaccharide glycal **36**. Deprotection of **36** and re-acetylation, provided the peracetate of the glycal, **39**. Epoxidation of **39** with dimethyldioxirane, solvolysis with allyl alcohol, and exhaustive saponification gave the allyl glycoside **40**, which now contains



Scheme 8. Synthesis of the Globo-H–KLH vaccine construct.

the access point to reach the fully functional vaccine. As before, ozonolysis of the allyl linker sets the stage ($\rightarrow$ **41**) for reductive coupling to KLH[22] thus yielding the immunogenically functional oligosaccharide–protein conjugate **42**. Carbohydrate:protein analysis[32] of such material routinely

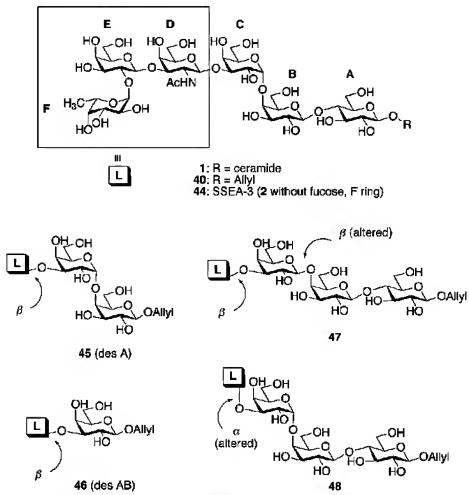


Scheme 7. Coupling of **30** with **35** and the last steps of the synthesis of MBr-1 antigen Globo-H **1**. a) MeOTf, Et_2O/CH_2Cl_2 (2:1), 70%; b) 1. DMDO, CH_2Cl_2; 2. **19**, $ZnCl_2$, THF, 53%; 3. Ac_2O, pyridine, 95%; c) H_2, Lindlar's catalyst, palmitic anhydride, EtOAc, 90%; d) 1. TBAF, THF; 2. NaOMe, MeOH; 3. Na/NH_3; 4. Ac_2O, pyridine, DMAP; 5. NaOMe, MeOH.

reveals approximately 350 Globo-H epitopes per molecule of KLH. Similarly, we investigated conjugates containing BSA where 17 carbohydrate units per protein were introduced (see **43**).

5.2. Synthesis of Truncated Structures Corresponding to Globo-H

With a viable synthesis in hand, we could extend the methodology to truncated versions of **1**. Such compounds might be more readily synthesized and might perhaps also be recognized by Globo-H directed antibodies.[45] We were much influenced in these synthetic studies by the fact that SSEA-3 **44** (Scheme 9), which lacks the terminal fucose residues, fails to bind the MBr1 antibody.[50, 51] Although the fucose moiety appears to be crucial, it was of interest to investigate which truncated or isomeric versions of **1** that contain the fucose group but lack residues from the reducing end of the carbohydrate domain would be recognized by the MBr1 antigen. Accordingly, through total chemical synthesis we assembled the allyl glycosides **45**–**48** by glycal assembly methods (Scheme 9).[44] The hexasaccharide core contained in **1** is maintained in compounds **47** and **48**, but in each case a glycosidic linkage is altered relative to the natural antigen. It should be noted that addressing the molecular recognition of the Globo-H antigen at such a detailed level (α versus β linkages) through truncated probe structures was only possible by the power of chemical synthesis and persistence of dedicated colleagues.



Scheme 9. Truncated versions of **40**.

With the four allyl glycosides available in sufficient quantities and rigorously defined in terms of structural integrity, we carried out binding studies to MBr1 antibody using MCF-7 cell lines as the targets for binding. Increasing amounts of glycosides (0.05 µg–500 µg) were used and the results from an individual inhibition experiment are demonstrated in Table 2. Compounds **45**–**47** show significant binding to MBr1, suggesting that the binding domain is localized in the CDEF ring system and that the terminal fucose is indeed critical for this particular antibody. In addition, the stereochemistry of the glycosidic linkage joining rings C and D also appears to be critical for binding to MBr1 antibody (No binding occurs with **48**). Apparently, once the properly configured CDEF domain has been presented, the nature of the glycosidic linkage joining rings B and C is of minimal consequence (see compound **47**). Thus, it does appear as though analogues of **2** can be recognized by the MBr1 antibody. However, immunoassessment in a polyclonal setting would be of greater importance.

Table 2. Inhibition of monoclonal antibody MBr1 binding to an MCF-7 cell line by synthetic antigens **45**–**48** as well as **40** and **44**.

	40	44	45	46	47	48
IC_{50} [µM][a]	16	> 500[b]	10	26	27	200

[a] Concentration for 50% inhibition. [b] IC_{50} was not reached.

5.3. Immunological Studies Pertaining to Globo-H Based Vaccines

We next turned our attention to a detailed immunocharacterization of this important Globo-H antigen and its analogues. Systems **42** and **43** were starting points for these investigations. As before, the initial stages toward development of a Globo-H based vaccine started with mouse vaccination studies[52] to confirm the immunogenicity of the synthetic glycoconjugates. The goal was to demonstrate that the synthetic vaccine conjugate combination activates the mouse immune system to produce antibodies that bind to human cancer cells expressing the epitope around which the carbohydrate region was organized.

Serological responses were analyzed by ELISA to determine antibody titers and the cell-surface reactivity of the resulting antibodies was assayed by flow cytometry and immune adherence assays. The ability of these sera to mediate complement lysis was also assessed. Both KLH and BSA conjugates (**42** and **43**, respectively) were initially investigated. As in the case of the Le^y based vaccine as well as other examples, the KLH-conjugate was superior in terms of immunogenicity when administered with the adjuvant QS-21.[53]

In the animal studies with vaccine **42**, sera from all mice provided high titer IgM and IgG responses against Globo-H antigen (median titers 1/128,000 and 1/2560, respectively; Figure 3). A critical finding was that these sera reacted with Globo-H positive cancer cells (MCF-7) and, in a control experiment, failed to react with the Globo-H negative B78.2 melanoma cells. Serological studies also revealed that these antibodies were highly effective at inducing complement-mediated cytotoxicity. The percentage of lysis with antibodies

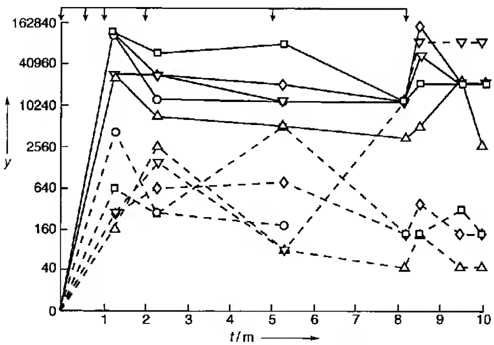


Figure 3. Time Course of the Antibody Titers in 5 Mice (◇, □, △, ○, and ▽) vaccinated with Globo-H–KLH conjugate **42** and QS-21. The reciprocal titer against Globo-H (determined by ELISA) is on the *Y* axis, the time *t* in months is on the *X* axis. ——: IgM titer, - - -: IgG titer. The vaccination times are indicated by the vertical arrows.

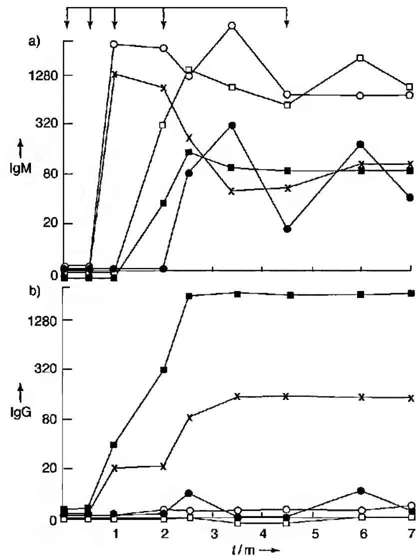


Figure 4. Time Course of the Antibody Titers in Five Patients (●, ×, ○, □, and ■) immunized with Globo-H–KLH conjugate **42** and QS-21. a) IgM titer; b) IgG titer. The reciprocal titer against Globo-H (determined by ELISA) is on the *Y* axis, the time *t* in months is on the *X* axis. The vaccination times are indicated by the vertical arrows.

induced by the Globo-H–KLH conjugate (**42**) was 48%. For comparison, reference experiments with monoclonal antibody MBr1 showed 72% complement-induced cytotoxicity. These immunogenicity results then provided the basis for further examination of the vaccine construct **42** in tandem with QS-21. To date, the carbohydrate moiety of the vaccine in question is the most complex synthetic antigen brought to the stage of clinical evaluation.

Upon discovering these favorable serological and cell-surface reactivity results in vaccination with mice, various protocols were proposed for the use of fully synthetic Globo-H vaccines in a clinical setting. Following resynthesis and reconjugation in an appropriate setting, a study was launched. The study involved prostate cancer patients who had relapsed following prostatectomy or radiation therapy.

It should be emphasized at this point, that in progressing from a murine to a human setting for vaccinations, potential risks had to be faced. Human sera and cell-surface glycoproteins present related structures (in the form of Lewis blood group determinants and, indeed, very low levels of globosides). Hence, in the human clinical setting, there are potential issues of immunotolerance or possibly autoimmune damage to be addressed. Such considerations were not pertinent to the mouse immunizations. A focused humoral antitumor response using the fully synthetic carbohydrate vaccine would be a satisfactory proof of principle.[54] These phase I trials were designed to test the safety of the vaccine and to investigate the proper doses of the vaccine conjugate and QS-21.

In the initial trial, five patients with progressive and recurrent prostate cancer received the KLH conjugate vaccine **42**, containing 30 µg of Globo-H plus 100 µg QS-21, according to defined clinical protocols.[55] Their sera were submitted for detailed analysis and evaluation. Post-vaccination IgG and IgM titers against Globo-H ceramide, as determined by ELISA, are depicted in Figure 4. Subsequent to vaccination, all five patients produced a strong IgM response, while two concurrently generated a high IgG response. The specificity of these antibodies for Globo-H in prostate cancer cell extract from tumor or biopsy, as well as breast cancer biopsy specimens, was analyzed by immune thin layer chromatography revealing that the post-vaccination sera recognized both synthetic and tumor derived Globo-H ceramide. By contrast, the sera failed to react with melanoma biopsy specimen extracts which contain various glycolipids but are Globo-H negative.

With post-vaccination sera available, inhibition assays were carried out to determine the specificity of the antiGlobo-H antibodies in the immunized patients. Synthetic Globo-H ceramide **1** and the structurally related antigens **45**–**48** (Scheme 9), which were previously prepared by total synthesis, were surveyed for inhibition by using IgM and IgG ELISAs and the results are shown in Figure 5. Although the truncated oligosaccharide isomers were recognized to some extent, synthetic **1** inhibits antiGlobo-H reactivity most efficiently. As a control, unrelated glycolipids and synthetic Le^y–allyl glycoside **15** showed no IgM response (Figure 5a). The antisera which demonstrated IgM ELISA activity in the IgG assay (Figure 5b). Both synthetic hexasaccharides **1** and **40** as well as pentasaccharide **45** effectively inhibited binding in this assay. Apparently, in the polyclonal setting, IgG antibodies from the two sera mainly recognize an epitope area encompassing on average five reducing terminal carbohydrate units.

The lack of recognition of Le^y antigen **15** and various probe structures, even in the polyclonal regime, also reflected a

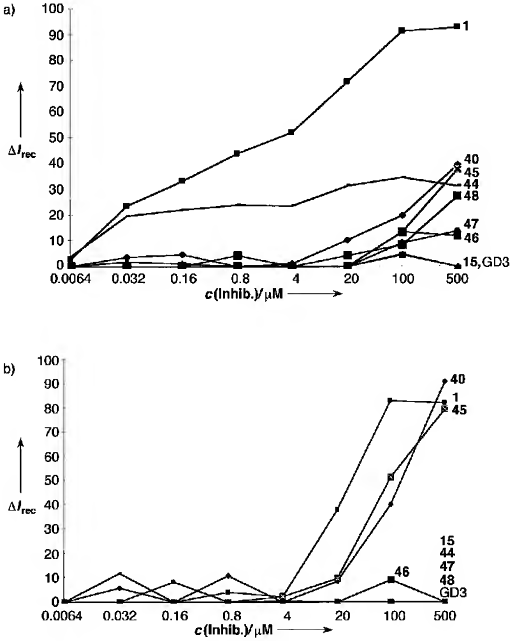


Figure 5. Analysis of the specificity of antiGlobo-H antiserum by inhibition assays. The ELISA reactivity of the serum with Globo-H ceramide when inhibited with compounds **1**, **15**, **40**, **44**–**49**, and GD3 (GD3 = NeuAcα2$\rightarrow$8NeuAc2$\rightarrow$3Galβ1$\rightarrow$4Glcβ1-1′-cer) is shown. a) IgM antibody response; b) IgG antibody response. (ΔI_{rec} = % Inhibition; c(Inhib.) = Concentration of inhibitor.)

disiplined response against portions of Globo-H. Clearly, the specificity for **1** arises from the difference in the structural and stereochemical connectivity of the antigenic subunits. The results demonstrate that a fucosylated structure is required for an optimal antiGlobo-H response (see structure **44**).

Encouraged by these findings, we pursued the critical question as to whether antibodies elicited by the KLH vaccine construct **42** could recognize the antigen in its natural context, that is to say, the cell surface. This type of recognition is obviously a crucial milestone in the progression and development of antitumor vaccines. Preincubation of sera with Globo-H positive (MCF-7) cell lines produced more than a 50% decrease in binding against Globo-H ceramide, indicating that much of the antibody was recognized and bound to the tumor cell surface. No decrease in binding activity was observed following incubation with Globo-H negative (SK-MEL-28) melanoma cells. Furthermore, cell-surface reactivity of the antiGlobo-H antibodies was tested by flow cytometry and the results showed an increase for IgM and, to a lesser extent, IgG antibodies.

A final element of the initial preclinical serological evaluation following vaccination with **42**, was to test for the ability of the resultant sera, containing antiGlobo-H antibodies to mediate complement-dependent cytotoxicity (CDC). Following relevant control experiments, results showed that three of the five post-vaccination sera exhibited strong CDC to MCF-7 cells. These results are also encouraging because complement-induced lysis of relevant cancer cells would seem to be associated with decreased tumor outgrowth and would favor longer survival.[7a]

With the successful demonstration that the vaccine construct **42** combined with the adjuvant QS-21 is safe in humans and induces specific antibodies against tumor cells which carry the same antigenic structure contained in the vaccine on their cell surface, we progressed to a larger patient trial. The full phase I trial was completed by 18 patients with progressive and reoccurring prostate cancer. The clinical details of the trial can be found in the literature.[56] All immunized patients exhibited good IgM responses against Globo-H confirming its immunogenicity in prostate cancer patients with a broad range of stages and tumor burdens. Pre- and post-immunization sera from the 18 patients were evaluated for the ability to mediate complement lysis. IgM antibodies induced in this trial were able to react with tumor cells as demonstrated by flow cytometry and, in nine cases, induced complement-mediated lysis of Globo-H expressing cell lines (Figure 6).

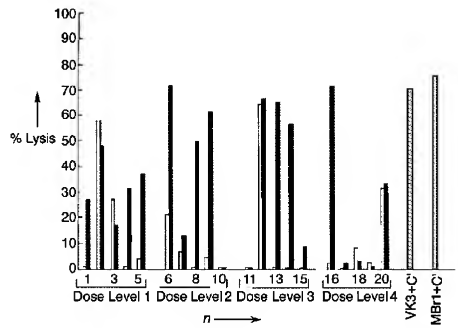


Figure 6. Complement lysis of MCF-7 tumor cells by pre- and post-immunization sera from 18 evaluable patients. Positive controls included MCF-7 cells with mAb VK9 (IgG) and MBr1 (IgM) with complement. White: before immunization, Black: after immunization, Grey: controls. n = Number of patients.

In addition to inducing proper immunogenicity, another important finding was advanced and addressed in the full patient sampling. Prostate cancer is, in principle, a unique disease to study because a highly specific biomarker, the Prostate Specific Antigen (PSA), is available. This allows the disease to be monitored at low tumor burdens where the vaccine therapies of interest to us are more likely to be effective. Measurement of PSA levels in several patients of this trial could indicate that a treatment effect could occur after completion of the vaccine therapy. Currently, the vaccination seems to bring about a decline of the slope in the plot of log PSA concentration versus time after treatment compared with values before treatment.

All patients showed PSA rises during the first 26 weeks of treatment, although in some cases PSA rate of rise appeared

to slow during the course of immunization. However, the potentially important, although preliminary, finding was that as patients continued to be observed for the six to nine - months post-treatment, favorable changes in the PSA slopes occurred in most patients who presented an initial non-metastatic state. By the criteria of declining PSA slopes, it could be argued that some actual treatment effect was occurring even after only three months. However, given the small sample size, these findings are highly tentative. An example of the PSA criterion is depicted in Figure 7.

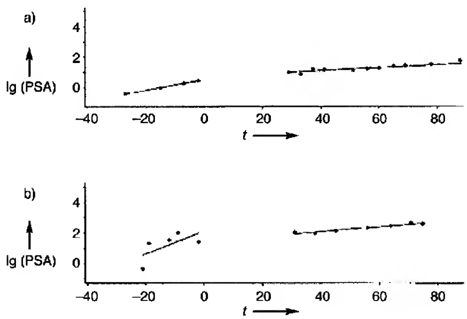


Figure 7. Decline of the slope of the logarithm of PSA concentration against time t after treatment of the patients in comparison with values before treatment. These patients continue to be radiographically free of disease more than 80 weeks after treatment and show stable PSA slopes. a) Slope before = 0.034, Slope after = 0.009; b) before = 0.075, after = 0.016.

Five patients from this trial, who continue to receive booster vaccinations, have stable PSA slope profiles in the absence of any radiographic evidence of disease after two years. In two of the five, the PSA slopes are decreasing. We emphasize that the concept of using PSA slope profiles for assessment of early treatment effects in biological therapies such as vaccines must await further evaluation in phase II and phase III trials and at present is not a reliable diagnostic.

As noted at the outset, Globo-H has been found to be expressed on a variety of tumors. Thus, we have hopes for use of a Globo-H based vaccine in other cancers as well. A vaccine containing the Globo-H antigen **42** has also been administered to breast cancer patients in a phase I clinical trial, according to defined clinical protocols. Data from the vaccinations are beginning to be correlated. Initially they appear to be encouraging at the serological level. A full report will be provided in due course.

With respect to further advancement of Globo-H based vaccines, we have obtained and organized clinical data to the point where we are planning a 200 patient trial on prostate cancer. Such a trial would use the fully synthetic vaccine construct **42** containing the Globo-H antigen, possibly in the context of a polyvalent setting which would include a menu of tumor antigens. With synthesis efforts proving increasingly successful,[57] a fully definitive 1000 patient trial involving Globo-H is also being organized to begin sometime this year.

6. Synthesis of the KH-1 Antigen

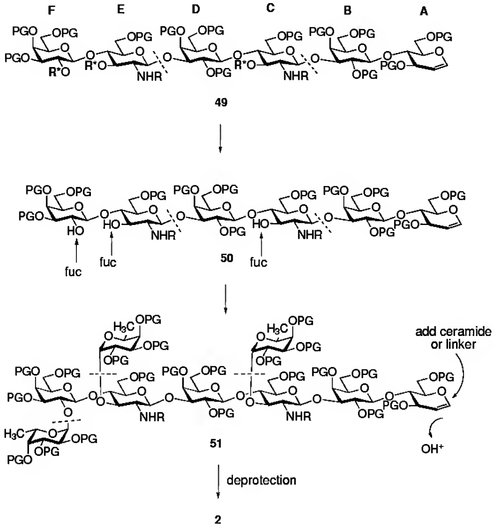
The glycolipid KH-1 **2** (see Figure 1) is perhaps the most formidable carbohydrate-based tumor antigen thus far characterized.[58] The antigen was isolated from human colonic adenocarcinoma cells by using antibodies generated against the classical Le^y determinant (**3** in Figure 1). System **2** has been present on the cell surface of all adenocarcinoma cells thus far studied. Furthermore, its presence has never been detected in normal colonic extracts. Obviously, the chances of success from the point of view of vaccinology will be higher with the greater specificity of the carbohydrate domain of the antigen.

Monoclonal antibodies were raised against this antigen and found to bind specifically to compound **2**. Based on these studies, Hakomori et al.[59] postulated that the KH-1 antigen is a highly specific marker for malignancy and premalignancy involving colonic adenocarcinoma. Recently, an X-ray crystal structure of an antitumor antibody BR96 in complex with the nonanoate ester derivative of Le^y tetrasaccharide was reported by Jeffrey et al.[60] The view of the antibody–Le^y complex provided by this determination suggested that the BR96 antibody has unused binding capacity which might also recognize structures larger than the Le^y tetrasaccharide (such as the KH-1 antigen).

We were attracted to the KH-1 antigen in terms of chemical synthesis for several reasons. At the outset, a total synthesis of **2**, and bioconjugatable precursors, would represent the construction of another extremely complex tumor antigen.[61] Embarking on such a task also provided the opportunity and setting to evaluate important strategic advances in terms of synthetic economy in oligosaccharide synthesis. Furthermore, our interests were not limited to **2**, but included congeners that would also be bioconjugated to the appropriate carrier systems.[62] Difficulties associated with isolation and separation of complex carbohydrates from human colonic cancer tissue have been such that compound **2** has not been available for evaluation. Only through total synthesis could workable quantities of such chemically defined complex systems be obtained to allow such evaluations.

Our synthesis of the KH-1 antigen **2** was reported in early 1998.[63] Not only was a total synthesis achieved, but the always important issue of strategy in oligosaccharide synthesis was suitably addressed as well. The synthetic plan put forth is outlined below.

Considerable thought was invested in gaining maximum relief from blocking group manipulations and obtaining optimal conciseness, a consequence which clearly becomes increasingly more difficult with increased branching and complexity in the oligosaccharide target. From these perspectives, we came to favor a plan that would build a hexasaccharide (**49**, Scheme 10), so differentiated in terms of its protecting patterns, as to allow for the simultaneous unveiling of the three free hydroxyl groups destined for fucosylation at a strategic point of our choosing. As the featured asset of our synthesis, the three fucosylations would then be conducted concurrently (see **50** → **51**). Requirement for three kinds of blocking groups in **49** was therefore necessary; one at the nitrogen centers, another for the three proposed fucosylation

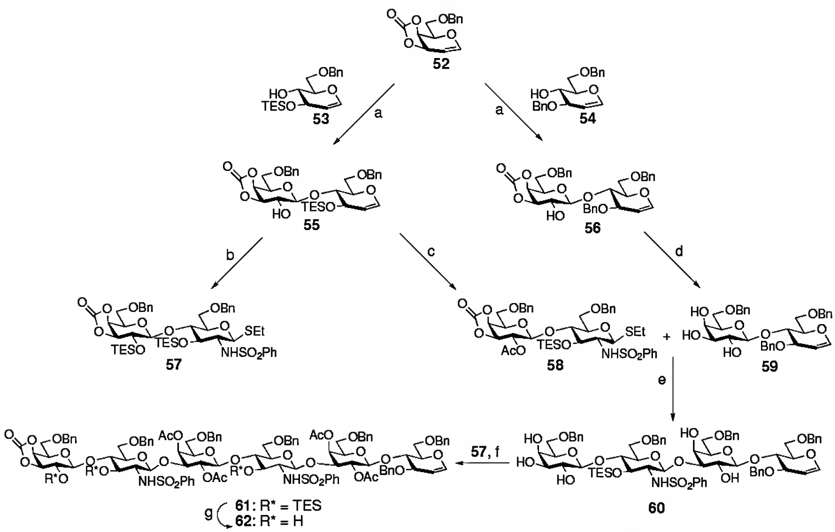


Scheme 10. Planned synthesis of KH-1 **2**. PG = generalized hydroxyl protecting group, R = nitrogen protecting group, R* = unique oxygen protecting group.

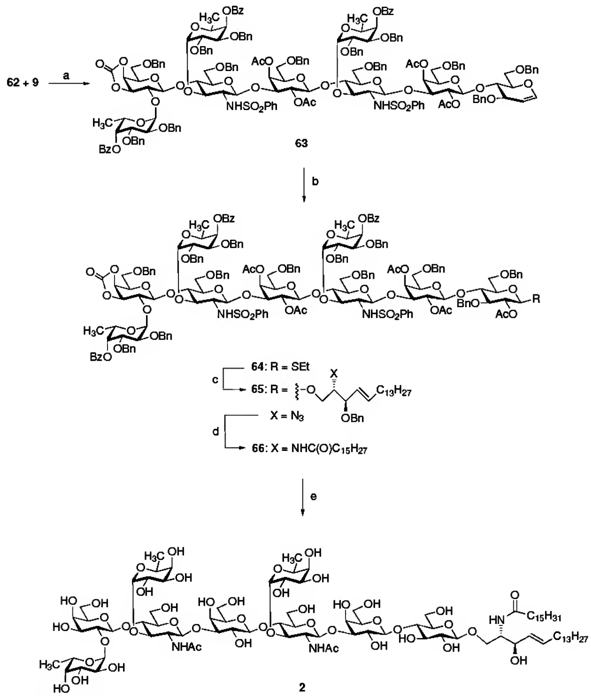
sites (R*) and a third for the remaining hydroxyls. As we have successfully demonstrated before, the terminal glycal functionality in **51** could then be used to provide access to the native KH-1 antigen **2** or to bioconjugates, en route to evaluatable antiadenocarcinoma vaccines. The synthesis based on such a plan is depicted in Scheme 10.

Suffice it to say, the construction of the hexasaccharide glycal which corresponds to structure **49** was a large undertaking in itself. The synthesis of differentially protected glycal **61** is presented in Scheme 11 without detailed discussion. It is possible to benefit from the conciseness of using glycal building blocks **52**, **53**, and **54** for rapid construction of large oligosaccharides, as shown in Scheme 11. Deprotection of **61** to yield **62** then allowed for the culminating stage of the plan to be put in place.

As hoped for, it proved possible to install the three α-L-fucose residues in a single synthetic step via fluoro donor **9** thereby affording the nonasaccharide glycal **63** in an exemplary yield (Scheme 12). From this point, the protocols required to reach **2** and related systems were much influenced by our earlier work in the Globo-H and Le^y series. The first step in the introduction of the ceramide side chain to reach the natural KH-1 antigen **2**, was the epoxidation of glycal **63**. While this reaction seemed to occur smoothly, attempts to use the epoxide directly as a glycosyl donor with acceptor **19** gave low yields of coupled products. Accordingly, we turned to the application of a recently developed variation of the glycal epoxy donor method.[64] This protocol started with epoxidation of **63** with dimethyldioxirane, followed by thiolation of the resulting epoxide and further acetylation, thus leading to acetate **64**. With the expectation of effective neighboring group participation by the C2 acetoxyl function available to



Scheme 11. Synthesis of hexasaccharide **62**. a) 1. DMDO, CH_2Cl_2; 2. **53** or **54**, $ZnCl_2$, THF, 65% for **55** and 55% for **56**; b) 1. TESOTf, Et_3N, CH_2Cl_2, 92%, 2. $I(coll)_2ClO_4$, $PhSO_2NH_2$, 4 Å molecular sieves, CH_2Cl_2, 90%; 3. LHMDS, EtSH, DMF, 90%; c) 1. Ac_2O, Et_3N, DMAP, CH_2Cl_2, 95%; 2. $I(coll)_2ClO_4$, $PhSO_2NH_2$, 4 Å molecular sieves, CH_2Cl_2, 90%; 3. LHMDS, EtSH, DMF 4. Ac_2O, Et_3N, DMAP, CH_2Cl_2, 85%; d) K_2CO_3, MeOH 80%; e) 1. MeOTf, DTBP, Et_2O/CH_2Cl_2 (2:1), 4 Å molecular sieves, 55%; 2. K_2CO_3, MeOH, 85%; f) 1. MeOTf, DTBP, Et_2O/CH_2Cl_2 (2:1), 4 Å molecular sieves, 60%; 2. Ac_2O, Pyridine, DMAP, CH_2Cl_2, 95%; g) TBAF/AcOH, 93%.



Scheme 12. Total synthesis of the KH-1 antigen **2**. a) $Sn(OTf)_2$, Toluene/THF (10:1), 4 Å molecular sieves, 60%; b) 1. DMDO, CH_2Cl_2; 2. EtSH, CH_2Cl_2, H^+(cat.); 3. Ac_2O, Pyridine, CH_2Cl_2; 60% over 3 steps; c) **19**, MeOTf, Et_2O/CH_2Cl_2 (2:1), 4 Å molecular sieves, 55%; d) Lindlar's catalyst, H_2, palmitic anhydride, EtOAc, 85%; e) 1. Na/NH_3, THF then MeOH; 2. Ac_2O, Et_3N, DMAP, CH_2Cl_2; 3. MeONa, MeOH, 70% over 3 steps.

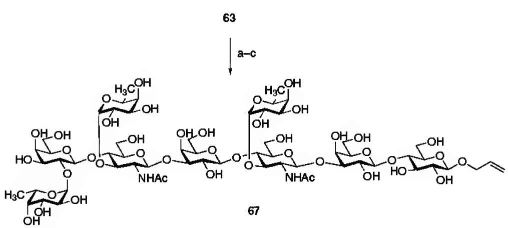
guide a β-glycosidation, compound **64** was treated with **19** under the agency of methyl triflate. This process indeed led to the formation of glycoside **65**, in greatly improved yield. From this point, the required methodology was quite familiar to us and the total synthesis of the KH-1 antigen **2** was completed as shown in Scheme 12.

For immunological studies, the allyl glycoside was again the goal system. For this purpose, we returned to glycal **63**. Removal of the protective groups followed by esterification gave the peractetylated glycal. Epoxidation under standard conditions and solvolysis with allyl alcohol installed the necessary spacer region. Exhaustive deacetylation yielded fully deprotected nonasaccharide **67** (Scheme 13) which was then conjugated to KLH as in the previous vaccine preparations.

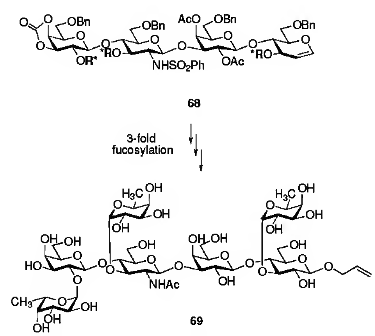
The structures of the products **2** and **67** were fully substantiated by mass spectroscopy, by self-consistent NMR analysis and, in the case of **2**, by comparison with the available fragmentary published data. A direct material comparison was not possible because of the nonfeasibility of obtaining the KH-1 antigen from natural sources. Owing to this synthesis, access to the KH-1 system is no longer an impassable problem.

In preparing for immunological investigations, it would be helpful to determine the specificities of various antibodies to the structural features of the KH-1 antigen. Toward that end, it was of interest to generate truncated structures in which segments of the molecule would be deleted. In our first such effort, we directed our attentions to a construct in which the three fucose residues, as well as an N-acetyl function, would be retained (**69**, Scheme 14). However, the reducing terminal *N*-acetyllactosamine substructure would be deleted. Again, we relied on a three fold fucosylation procedure to install the required linkages and the remaining steps in the conversion of fucosylated **68** to construct **69** ran parallel to those used in the synthesis of **67** (Scheme 14). Indeed, the strategy of threefold fucosylation to achieve conciseness in the synthesis was successfully demonstrated.

With all of the goals from a chemical standpoint realized, the focus on the KH-1 antigen shifted to issues of immunology and vaccinology. Mice were immunized with the fully



Scheme 13. Synthesis of allyl glycoside **67**. a) 1. Na/NH_3 then MeOH; 2. Ac_2O, pyridine, DMAP; b) 1. DMDO, CH_2Cl_2; 2. allyl alcohol; c) NaOMe, MeOH, 60% over 3 steps.



Scheme 14. Synthesis of a truncated version of **2**.

synthetic KH-1 vaccine containing **67** which was administered together with QS-21 as an immunoadjuvent. The mice responded to the vaccine with strong antibody titers. Studies of the immunological properties of the murine antibodies so elicited, with a view to possible clinical trials, are well underway and the results will be disclosed shortly.[65]

7. Future Vaccine Prospects

7.1. Synthesis of the Small Cell Lung Carcinoma Antigen Fucosyl GM_1

In bringing the synthesis of this review to a close, we update the reader on our recently completed, but as yet unpublished, total syntheses of the fucosyl GM_1 and N3 antigens. Clearly, in approaching these studies we were much influenced by our previous successes. The biological and chemical rational for addressing these targets will be described only briefly. They integrate these syntheses into the context of our ongoing vaccinology program.

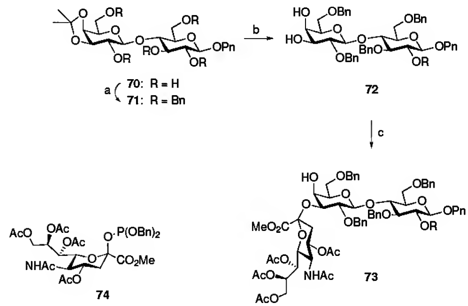
The glycolipid fucosyl GM_1, **7** (Figure 1), has been identified as a highly specific marker associated with small cell lung cancer (SCLC) cells.[66, 67] At the outset of our investigations, no total syntheses of **7** had been accomplished. Because of the tumor specificity which antigen **7** displays,[68] we were encouraged not only to perform a total synthesis of the requisite hexasaccharide core, but to devise a synthesis capable of generating material for possible clinical evaluation as well. Our recent efforts towards the synthesis of the pentenyl glycoside of fucosyl GM_1 are described below.[69]

Recognition that the three saccharides at the nonreducing end of **7** are identical to the DEF portion contained in Globo-H **22** (Scheme 4) disposed us to think in terms of another [3+3] coupling. Using this disconnection however, would require a potentially difficult merger using a trisaccharide, which contains a protected sialic acid residue, as the acceptor. We assumed this risk mindful of our earlier precedent, however imperfect, from the Globo-H series. We also drew inspiration from another precedent from our laboratory, practice in the assembly of GM_1 using a related sulfonamidoglycosylation.[70] For vaccine development involving fucosyl GM_1, we envisaged the installation of a functionalized glucopyranoside at an early stage in the synthesis, rather than relying on manipulation of the fully mature oligosaccharide.

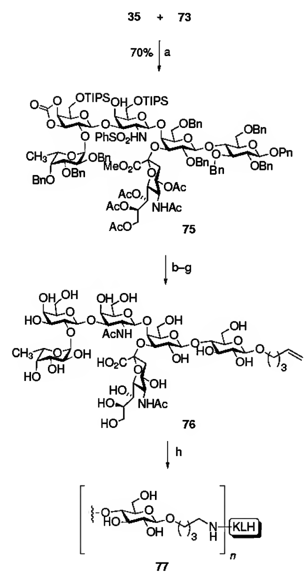
The synthesis of the requisite trisaccharide acceptor is shown in Scheme 15. Pentenyl lactoside[71] was converted to its thermodynamic C3′,C4′-acetonide to give **70** and subsequently perbenzylated to give the differentially protected **71**. Removal of the acetonide protecting group revealed two hydroxyl groups to give **72**. Diol **72** would successively act as an acceptor in, first, a sialylation reaction with **74** and, secondly, in the [3+3] coupling. Reaction of phosphite donor **74**[72] with TMSOTf in the presence of **72** gave the trisaccharide **73** as the only observable trisaccharide product.

Trisaccharide donor **35** is readily accessible from the Globo-H synthesis (see Scheme 6). Coupling of **35** to **73** under the action of MeOTf proceeded to give the hexasaccharide **75** in good yield, again demonstrating the power of the azaglycosidation sequence (Scheme 16). Hexasaccharide **75** was deprotected under standard conditions to yield the pentenyl glycoside of fucosyl GM_1 **76**. Our assignment of the structure **76** was based on NMR analysis of intermediates en route to the final structure and was supported by high resolution mass spectrometry. This constituted the first total synthesis of the fucosyl GM_1 hexasaccharide core.

Also of notice in this synthesis was that the pentenyl glycoside modification allowed for a much more efficient synthesis of potential conjugation precursors because late



Scheme 15. Synthesis of **73**. a) BnBr, NaH, DMF, 84%; b) AcOH/H_2O (4:1), 90%; c) **74**, TMSOTf, EtCN, 4 Å molecular sieves, −40°C, 75%.



Scheme 16. Synthesis of **77**. a) MeOTf, CH_2Cl_2/Et_2O, 0°C, 70%; b) TBAF, AcOH, THF; c) NaOMe, MeOH; d) NaOH, THF; e) Na/NH_3, THF, −78°C, then MeOH; f) Ac_2O, pyridine, DMAP, CH_2Cl_2, 46% over 5 steps; g) Steps c and d, 96%; h) 1. O_3, MeOH; Me_2S; 2. KLH, $NaCNBH_3$, phosphate buffer.

stage adjustments (in some cases rather costly) were effectively avoided. We are actively pursuing this strategy for immunoconjugation in other cases.[73]

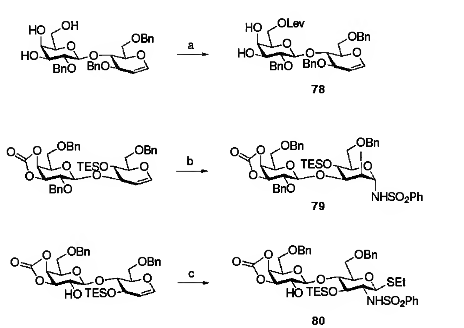
Fucosyl GM_1 pentenyl glycoside **76** has been immunoconjugated to carrier protein KLH to give glycoconjugate **77**. Importantly, as was shown with this result, the pentenyl linker apparently served as well as the allyl linker for conjugation purposes. Initial control studies with **76** and mouse vaccination studies with KLH-conjugate **77** are currently underway.

7.2. Synthesis of the N3 Antigens

The presence of anti-N3 antibodies in the serum of early stage gastrointestinal (GI) cancer patients has been shown to correlate with the development of GI cancer. We, as well as others,[74] have high hopes that a suitably conjugated version of this antigen could be used to detect the onset of even minuscule amounts of the N3 antibody. A group in our laboratory has recently completed a total synthesis of the N3 major and N3 minor antigens **4a** and **4b** (Figure 1), as well as the more biomedically versatile allyl glycosides.[75]

As described earlier (Scheme 12), central to our synthesis of KH-1 was the concept of polyfucosylation of a suitably protected acceptor. The structural features of N3 antibodies (namely a high degree of branched fucose residues) suggested that a similar approach could be invoked on a hexasaccharide core. It was also soon recognized that the required hexasaccharide core could be obtained from three disaccharide building blocks using glycal assembly methods.

The challenge of appropriate protection and deprotection sequences played a determining role in achieving this goal. The disaccharide building blocks needed for N3 major and N3 minor antibodies are shown in Scheme 17. Disaccharide **78** contains a differentiating levulinate (4-oxopentanoate) protecting group at C6′, formed by a selective acylation. Synthesis of the iodosulfonamide **79** was achieved from the corresponding glycal, itself available from glycal building blocks. The required differentiating protecting group in **79** is the C4 silyl ether. Finally, donor **80** originated from the corresponding



Scheme 17. Synthesis of the disaccharide building blocks **78**–**80**. a) Levulinic acid, CMPI, Et_3N, dioxane, 91%; b) $I(coll)_2ClO_4$, $PhSO_2NH_2$, CH_2Cl_2, 90%; c) 1. Step b, 94%; 2. LHMDS, EtSH, DMF, 85%.

lactal derivative and contains a C3 silyl ether for appropriate unmasking.

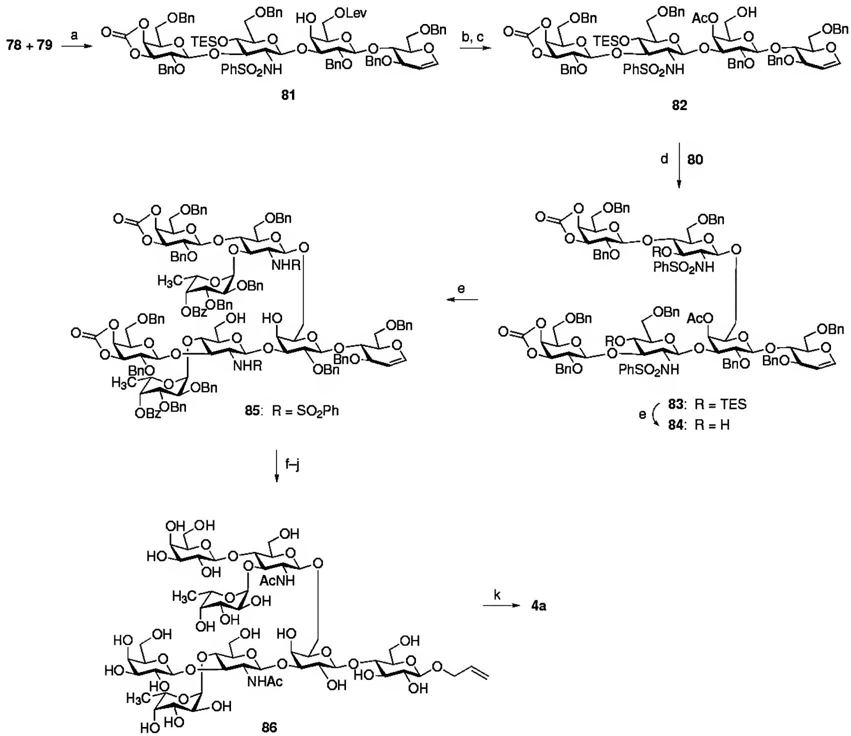
We relate here the synthesis of the major N3 antigen **4a**. (Scheme 18). Iodosulfonamide donor **79** was coupled directly to the stannane derived from **78** to yield the tetrasaccharide **81**. To allow for incorporation of the second disaccharide subunit, the resulting free hydroxyl group was acetylated, followed by the selective unmasking of the C6′ levulinate. Coupling of **82** with donor **80** gave the hexasaccharide core **83** as a glycal. Clearly, a significant amount of design was required to reach this structure. This concept was exemplified by subsequent removal of the two silyl protecting groups in **83** to reveal the two new acceptor sites for the expectant fucosylation. Indeed we were pleased when bisfucosylation of diol **84** using fluoro donor **9** provided the fully protected octasaccharide **85**. The sequence of deprotection, epoxidation, solvolysis with allyl alcohol and deacetylation were performed to yield bioconjugatable allyl glycoside **86**. Removal of the allyl group completed the synthesis of **4a**, as a mixture of α- and β-isomers. Following a similar course, the minor N3 antigen **4b** and its allyl glycoside were also synthesized starting from disaccharide blocks **78** and **80**. Further evaluation of the fully synthetic N3 antigens is in progress and the results of these inquiries will be disclosed in due course.

8. Antigen Clusters as Carbohydrate-Based Vaccines

As a second-generation synthetic investigation in our program, we began to undertake the development of synthetic methodology of general applicability for the preparation of glycopeptide-based vaccine constructs that mimic the cell-surface of tumor cells. Our initial focus has been on mucin-related O-linked glycopeptides. The remainder of this account will emphasize our efforts to synthesize and demonstrate that trimeric clusters of glycoepitopes, appropriately bioconjugated, are immunogenic as judged by antibody production and that these clustered constructs warrant further investigation as glycopeptide-based anticancer vaccines. In fact, one construct is beginning phase II human clinical trials.

Mucins, which comprise a family of large glycoproteins expressed on cells of epithelial tissues, carry large glycodomains in clustered modes.[76] Mucin amino acid sequences possess a very high percentage of serine and threonine residues, often found in contiguous arrays ranging in number from two to five. In most cases, the details of the occupancy of such blocks of serine and threonine subunits are not known in detail.[77] Despite a large variety of mucin glyco-structures, the modality wherein the first residue, an *N*-acetylgalactosamine moiety, is linked to a serine or threonine residue via an α-linkage appears to be broadly conserved (Figure 8). The glycophorin family of α-O-linked carbohydrates is a well-known and studied class of tumor antigens.

The Tn antigen **5** (Figure 1) represents the simplest member of the glycophorin family, as shown in Figure 8. This antigen, as well as the related Thomsen–Friedenreich disaccharide (TF) antigen **6** (Figure 1) is quite common in



Scheme 18. Synthesis of **4**. a) $(Bu_3Sn)_2O$, benzene, **78**; then **79**, $AgBF_4$, THF, 4 Å molecular sieves, 67%; b) Ac_2O, DMAP (cat.), pyridine, 88%; c) H_2NNH_2, pyridine, AcOH, 83%; d) TBAF, AcOH, 93%; e) **9**, $Sn(OTf)_2$, DTBP, 4 Å molecular sieves, toluene/THF (10:1), 76%; f) Na/NH_3, then MeOH; g) Ac_2O, pyridine, DMAP, 58% over 2 steps; h) DMDO, CH_2Cl_2; i) allyl alcohol, 41% over 2 steps; j) NaOMe, MeOH, 88%; k) $PdCl_2$, wet MeOH, quantitative yield.

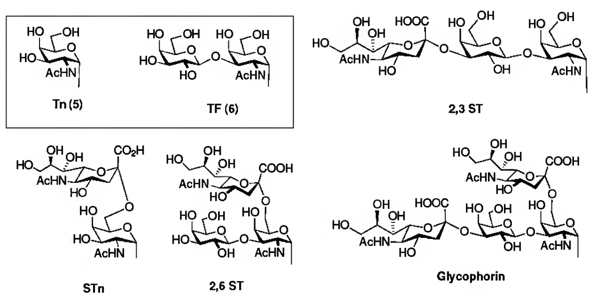


Figure 8. The glycophorin family of α-O-linked antigens.

carcinoma malignancies, particularly of the colon and prostate.[78] These simple carbohydrate antigens have been synthesized and their immunogenicity in conjugate vaccines confirmed.[79] For example, antibody titers against STn have been reported to correlate with improved prognosis in breast cancer patients.[80] Comparable studies with more complex carbohydrates have rarely been described, thus we developed a clear interest in the synthesis of large clustered forms of these antigens.

9. The Cassette Approach

The Tn and TF antigens have previously been prepared by a number of methods,[81] however at the time of our investigations, there remained a long-standing problem in glycopeptide synthesis.[82] The crux of the difficulty has been the problematic character of synthesizing carbohydrate domains O-linked to the key amino acids, serine and threonine, with strong stereochemical control in the formation of the α-glycosidic linkage. After much study with more complex targets, we were unable to provide a generally reliable protocol that would deliver the required amino acid to fully mature substrates of our choosing with high α-selectivity.

The glycopeptide assembly method that we have consequently developed and come to rely on is that of "cassette" modality rather than a maximally convergent approach.[83] In the cassette strategy, we build an *N*-acetylgalactosamine synthon stereospecifically O-linked to a serine (or threonine) residue with a differentiable acceptor site on the GalNAc. This construct serves as a general insert (cassette) that is joined to a target saccharide bearing a glycosyl donor function at its reducing end. In this way, we avoid the need for direct coupling of the serine side chain hydroxyl group to a fully elaborated, already complex saccharide donor. The clear advantage of this method is the need to only solve the very difficult O-linkage problem once for a given "reducing end" and to exploit that capability for building on the desired clustered system. Figure 9 demonstrates the logic of the cassette strategy. It is with this strategy in mind that we set out to synthesize clustered Tn antigen immunogenic structures and also put the cassette model to the test with the simplest case of clustered TF antigenic structures. With a successful venture, clusters containing other members of the glycophorin family of antigens would ultimately be within reach.

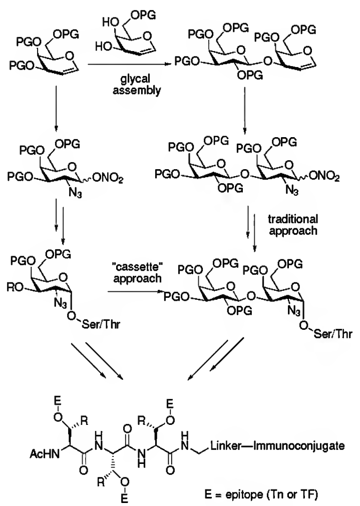
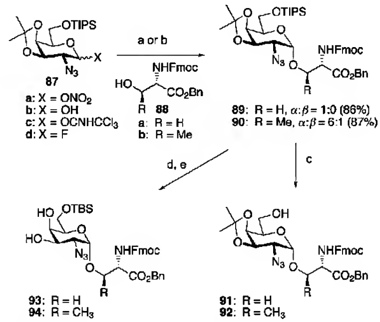


Figure 9. The cassette method for glycopeptide synthesis.

The most appealing approach we have found to the desired α-O-linked cassettes is shown in Scheme 19.[84] Donors **87c** and **87d** were generated from anomeric alcohol **87b**, itself available by azidonitration[85] of the corresponding glycal to give **87a**. In the case of serine derived acceptor, the glycosylation ratio apparently gave only the α-product **89**. In the synthesis of the threonine product **90** only a small amount of β-product was noted. Both preparations proceeded in excellent yield. Thus, the idea then emerged to use **89** and **90** as general inserts (cassettes) to be installed toward the end



Scheme 19. Synthesis of cassettes **91**–**94**. a) R = H: **87c**, TMSOTf, THF, −78°C; b) R = Me: **87d**, $[Cp_2ZrCl_2]$, AgOTf, CH_2Cl_2, c) TBAF, AcOH, THF, 94–100%; d) I_2/MeOH (63–81%); e) TBSCl, imidazole, DMF, 64–85%.

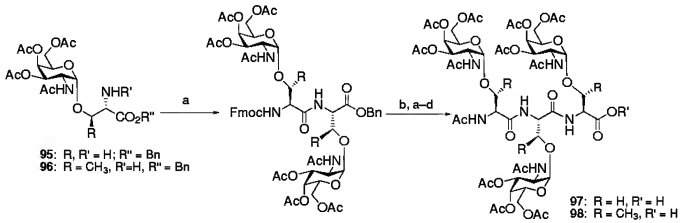
of a complex synthesis. However, to implement this strategy, a variety of orthogonally protected modules for further use as glycosyl acceptors were required. Accordingly, with the transformations shown in Scheme 19, ready access to position 6 acceptors **91** and **92**, or position 3 acceptors **93** or **94** was achieved.

10. Tn-Clustered Immunoconjugates

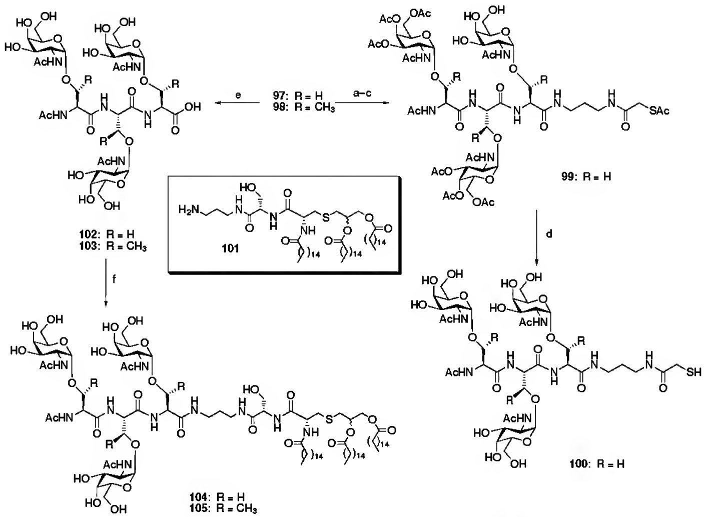
10.1. Synthesis of Tn-Clustered Immunoconjugates

With respect to synthesizing a Tn cluster, we turned to compounds **95** and **96**.[86] Due to the lack of reliable information regarding which serine or threonine residues within a contiguous array constitute an optimal epitope, a sequence of three consecutive Ser/Thr residues was chosen for initial evaluation.[87] Following standard peptide coupling procedures the synthesis of the trimeric clusters **97** and **98** was completed (Scheme 20). In bringing the synthesis to completion, the amino terminus was capped and the remaining carboxy terminus was freed to allow for further modification. For the purposes of creating a functional vaccine, such modification included conjugation to a synthetic lipopeptide as the immunological activator, or to an immunogenic carrier protein as in previous studies.

The two pathways that were followed for eventual conjugation are shown in Scheme 21. The first pathway involved attachment of a suitable linker for conjugation with a carrier protein. In a slightly different protocol with regard to our previous constructs, we investigated the mercaptoacetamide unit for this purpose.[88] Acid **97** was coupled with *tert*-butyl-*N*-(3-aminopropyl)carbamate with the agency of IIDQ. This step was followed by removal of the previously introduced Boc cap, and coupling with S-acetylthioglycolic acid pentafluorophenyl ester. The resulting fully protected glycopeptide **99** was then subjected to methanolysis under carefully controlled conditions (pH ≈ 9, degassed MeOH) to give clustered **100** which was now ready to be conjugated to the appropriate carrier (KLH or BSA).



Scheme 20. Synthesis of the trimeric Tn cluster. a) 95/96 (where R' = Fmoc, R" = H), IIDQ, CH_2Cl_2, 85–97%; b) 20% morpholine in DMF, 90–100%; c) Ac_2O, CH_2Cl_2, 70–76%; d) Pd/C, H_2, MeOH, H_2O, 85–95%.



Scheme 21. Preparation of the clustered immunoconjugates 100, 104, and 105. a) $H_2N(CH_2)_3NHBoc$, IIDQ, CH_2Cl_2; b) TFA, CH_2Cl_2; c) SAMA-(OPfp), DIEA, CH_2Cl_2, 81%; d) NaOMe, MeOH (degassed), 85%; e) NaOH, MeOH, 95%; f) 101, NHS, EDC, DMF, DIEA or HOAt, HATu, DMF, collidine, 35–40%.

For the synthesis of a fully synthetic lipopeptide, we followed the method of Tokoyuni et al. for attaching tripalmitoyl-*S*-glycerylcysteinylserine (Pam_3Cys 101).[89] Pam_3Cys has proven to be a potent macrophage and B lymphocyte activator, and has been pioneered for purposes similar to ours by Tokoyuni and co-workers with one to three epitopes of serine Tn.[90] First, careful saponification of 97 or 98 with NaOMe/MeOH gave the fully deprotected glycopeptides 102 or 103. Coupling with amine 101 using either the NHS or HOAt/HATU methods[91] then afforded glycolipids 104 or 105.

10.2. Discussion of Early Immunological Results

The initial experiments were to evaluate the antibody response to vaccination of mice with either Tn(cluster)-lipopeptide 104 or more conventional Tn(c)-KLH or -BSA conjugates. The preparation of these conjugates started with the previously described 100, which was covalently linked with carrier proteins.[92] For KLH, about 317 clusters per protein were introduced, while BSA showed only 7 clusters per protein.

These conjugates plus the adjuvant QS-21, Tn(c)-pam 104 in intralipid, or 104 in intralipid plus QS-21 were used to vaccinate groups of five mice. All of these constructs proved to be immunogenic. The median IgM and IgG ELISA titers against Tn(c)-pam in sera are shown in Table 3. Although sera of mice immunized with 104 in conjunction with QS-21 failed to show strong reaction, construct 100 conjugated with KLH induced high IgM and moderate IgG titers.

The cell surface reactivities of anti-Tn(c) antibodies were also evaluated using Tn(c) positive LS-C colon cancer cells and Tn(c) negative LS-B colon cancer cells. As before, measurements involved flow cytometry assays and complement-dependent cytotoxicity (CDC) assays. Sera from mice vaccinated with 100-KLH or 100-BSA with QS-21 showed clear IgM reactivity with LS-C colon cancer cells by flow cytometry. Significant IgG reactivity was also seen.

Again, the arguments for a progression into human clinical trials were much augmented with these studies. A phase I human trial using 100-KLH in a prostate cancer trial has just been completed and has produced extremely positive serological results. As a result, the synthetic construct 100-KLH is now planned to enter phase II clinical trials in a multivalent context.

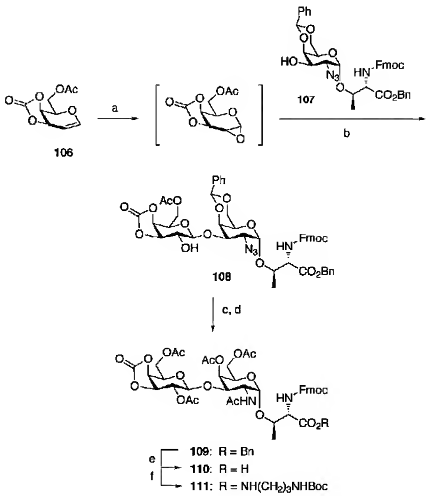
Table 3. ELISA Antibody Titers Against Tn(c).[a]

Mice	Vaccination			
	Before		After 3rd immunization	
Vaccine	IgM	IgG	IgM	IgG
104	0	0	1350	150
104 + QS-21	0	0	1350	50
100-KLH	0	0	12150	450
100-BSA	0	0	1350	150

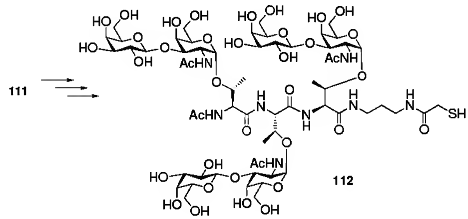
[a] All titers are medians for groups of five mice.

11. Synthesis of Clustered TF Antigen

With the successful immunization of clustered antigens well demonstrated, we next directed our attention to the synthesis of clustered TF disaccharide. Early efforts revealed that constructing the α-O-linkage on the fully mature disaccharide was plagued by poor selectivity. Thus, an ideal situation for application of the cassette methodology presented itself. To implement the strategy, we turned to our glycal assembly logic to simplify the construction. The idea was to use the glycosylated amino acid with the required α-O-linkage in place as the glycosyl acceptor. As shown in Scheme 22, the epoxide derived from glycal **106** proved to be a powerful donor in reaction with cassette **107** to afford the β-linked disaccharide **108**. At this point, we decided to attach the protected diamine linker first (see **111**), and then proceed on to clustering.[93] Similar processing of intermediates as in the synthesis of the Tn cluster produced the trimeric TF cluster **112** (Scheme 23).

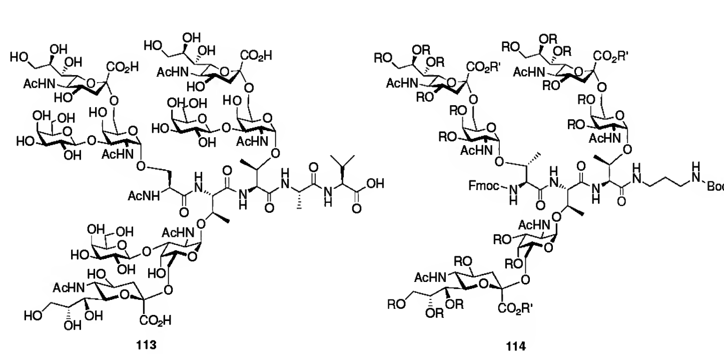


Scheme 22. Cassette coupling to provide TF disaccharide **108**. a) DMDO, CH_2Cl_2, 0°C; b) **107**, $ZnCl_2$, THF, −78°C→RT, 97%; c) 1. $AcOH/H_2O$ (4:1), 70°C, 3 h; 2. Ac_2O, DMAP, TEA, CH_2Cl_2, 93%; d) $CH_3C(O)SH$, 19 h, 87%; e) Pd/C, H_2, 2 h, quantitative yield; f) HOAt, HATU, collidine, DMF, 84%.



Scheme 23. Synthesis of the TF cluster.

Following the methodology provided in the syntheses described here, our cassette strategy in combination with the glycal assembly methods have also culminated in the synthesis of two other clustered motifs containing glycophorin family members. Structures **113**[94] and **114**[95] containing the 2,6-STF[96] and STn groups,[97] respectively, have been synthesized (Scheme 24). Conjugation and immunological evaluation of these constructs is currently well under way.



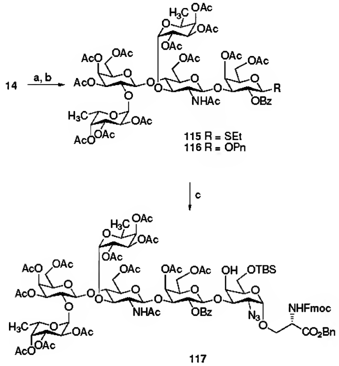
Scheme 24. 2,6-STF and STn clusters **113** and **114**.

12. The Lewis^y-Clustered Glycopeptide

12.1. Synthesis of Lewis^y-Clustered Glycopeptide

The complexity of many issues to be overcome in pursuit of a fully synthetic homogeneous blood group determinant in a clustered setting presented a clear challenge to the science of chemical synthesis. We embarked on this challenge by confronting the synthesis of the clustered Lewis^Y determinant.[98] As always with glycopeptide clusters, we were also interested in providing for installation of a flanking sequence through the carboxy terminus culminating in the immunostimulatory Pam_3Cys moiety. Given the range of protecting groups necessary to support such a synthesis, this requirement proved to be a major element of the undertaking.

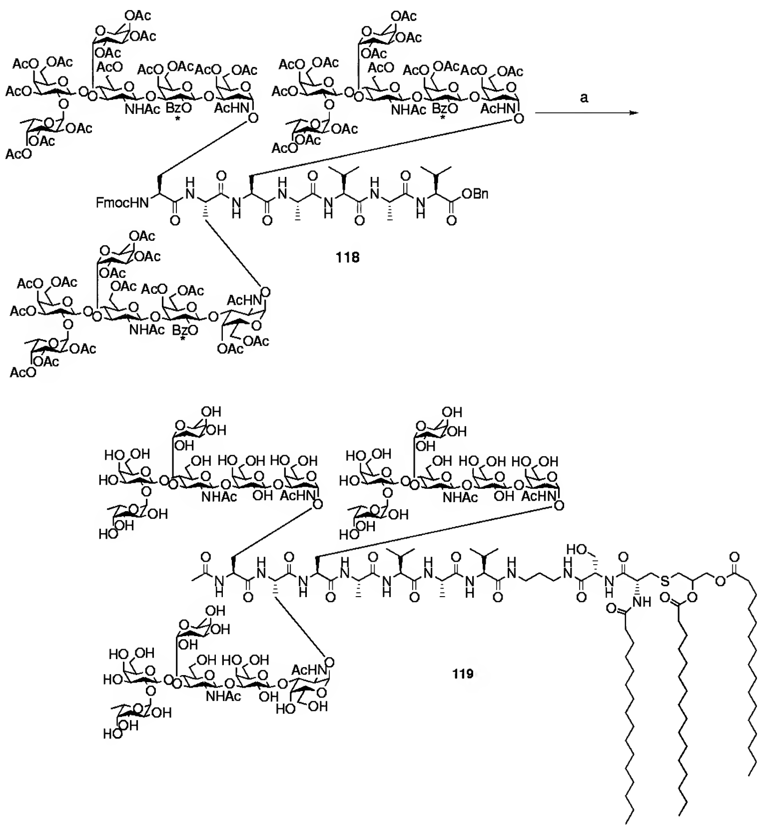
In keeping with the cassette strategy, we returned to the glycal **14** which contains the Le^y specificity (Scheme 1). Upon suitable activation of the glycal for glycosylation, serine cassette **93** (Scheme 19) was called upon to play the acceptor. As shown in Scheme 25, glycal **12** was epoxidized in the usual way and converted to the thioethyl donor **115**. In subsequent studies, the pentenyl glycoside donor **116**[99] was also prepared by reaction of the epoxide with pentenyl alcohol.[100] The C2



Scheme 25. Cassette coupling to give the Le^{Y} pentasaccharide. a) 1. DMDO, CH_2Cl_2, 0°C; 2. EtSH, TFAA, 40–50%, or PnOH, $ZnCl_2$, THF, −78°C, 83%; b) BzCl, pyridine, CH_2Cl_2, DMAP, 40–50% for R = SEt, 97% for R = OPn; c) **93**, NIS, TfOH, 4 Å molecular sieves, CH_2Cl_2, 79% for R = SEt, 83% for R = OPn.

benzoate protecting group was installed to minimize orthoester formation and to allow for the desired β-selectivity in the glycosylation. In the [5+1] cassette coupling event, using NIS/TfOH for promotion,[101] hexasaccharide **117** bearing the required serine α-O-linked to the complex carbohydrate domain was obtained in outstanding yield.

The cassette linked construct **117** was then advanced through the peptide assembly phase. Iterative peptide couplings, guided by our previous cluster syntheses, provided the trimeric cluster **118** (Scheme 26). In advancing **118** to become a functional immunoconjugate, the Fmoc-protecting group was removed and the free amine was capped by acetylation. Hydrogenolytic cleavage of the benzyl ester then exposed the C-terminal carboxyl. In the culminating global deprotection step, treatment with hydrazine hydrate in methanol smoothly cleaved the acetate and benzoate esters to afford the fully deprotected glycopeptide. The success of the hydrazinolysis step was crucial, since the benzoate protecting groups on the three galactose spacers (see asterisks) insulating the determinant from the serine residues, had resisted typical deprotection conditions.[102] Finally, the lipid amine (Pam_3Cys) **101** was

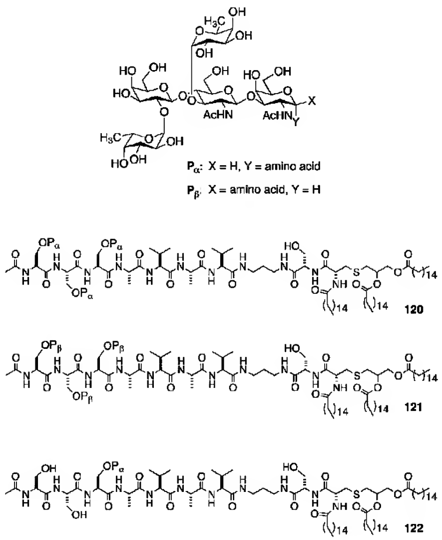


Scheme 26. Conversion of heptapeptide **118** to antigenic construct **119**. a) 1. Morpholine, Ac_2O; 2. H_2, Pd/C; 3. hydrazine hydrate; 4. **101**, HOAt, HATU.

coupled to the acid terminus of the heptapeptide under the conditions shown to afford the synthetic antigenic construct **119**, thus completing this total synthesis of a mucin like cluster of fully synthetic Le^y epitopes.

12.2. Immunogenic Consequences of Clustered Le^y Glycopeptide

Through total synthesis efforts, three additional pentasaccharide-based constructs lacking the internal galactose were prepared through a conceptually related route (Scheme 27): a trisubstituted lipopeptide **120** which retains the α-GalNAc linkage of **119**, a similar construct with a β-linked GalNAc **121**, and a singly Le^y-substituted lipopeptide **122**. In this route, when we did not follow the cassette logic, the glycopeptide



Scheme 27. Synthetic Clustered Probe Structures.

synthesis was nonstereospecific and therefore allowed for isolation of both α- and β-glycosyl serine stereoisomers. Thus, through total synthesis, including nonstereospecific total synthesis, we had the means to probe the cell surface architecture of tumor cells. The immunological evaluations conducted with the hexasaccharide construct **119** and the series of pentasaccharide constructs **120**–**122** were designed to make comparisons between the isomeric structures.

An ELISA was used to determine the immunological reactivities to anti-Le^y antibody 3S193[103] of lipoglycopeptide constructs containing Le^y **120**–**122**, as well as the control compound Le^y-ceramide **3** (Figure 10). This antibody had been elicited by tumor cells that presumably display the cell surface mucin motif. Of the synthesized constructs, the α-O-linked hexasaccharide **120** and the β-O-linked glycopeptide containing Le^y **122** were the most reactive and were com-

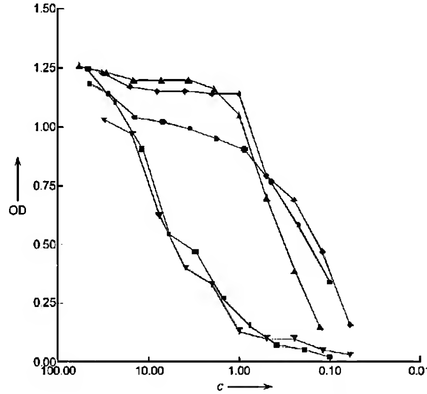


Figure 10. Reactivity of synthetic Le^y hexa- and pentasaccharide lipoglycopeptides with mouse anti-Le^y monoclonal antibody 3S193 as determined by ELISA. ◆: **119**, ■: **120**, ▲: **121**, ▼: **122**, ●: Le^y-ceramide (**3**). The optical density O is shown against the antigen titer in ng per test sample.

parable to the Le^y-ceramide control **3**. The α-O-linked monomer and trimeric constructs **122** and **120**, respectively, showed similar reactivity to one another, but were significantly less well bound than the control. These results suggest that the constructs having a β-linkage for the attachment of the terminal pentasaccharide most closely resemble the tumor-expressed, cell-surface Le^y antigen against which the antibody 3S193 was elicited.

In the next phase, mice were immunized with the Le^y-pentasaccharide constructs without adjuvant and the antisera were tested against Le^y-ceramide, Le^y-mucin, and Le^y-expressing tumor cells to examine the effects of antigen structure on immunogenicity and the tumor cell reactivity of the antibody response. Clustering of the glycodomain was found to be crucial for antibody production against natural substrates. The α- and β-O-linked trimeric structures **120** and **121** are highly immunogenic with levels of antibody response to Le^y-ceramide and Le^y-mucin comparable to Le^y-KLH, whereas the immunological response of the monomeric construct **122** to the same targets was poor. The same trend was observed in FACS analysis of cell surface reactivity. Antisera produced against the clustered motifs each bound Le^y positive tumor cells more efficiently than the monomeric structure (74 % versus 54 %).

Interestingly, the natural glycosidic linkage to the amino acid that is found in mucin glycoproteins is not critical for antibody production to Le^y-bearing glycolipids and mucin, as **120** is equally as immunogenic as **121**. Also noteworthy is that antibody response to the lipopeptide constructs was primarily IgM, whereas Le^y-KLH produced IgG as well as IgM antibodies. It appears that the Pam$_3$Cys immunomodulating unit stimulated only B cells in this study. Nonetheless, this study represents the first demonstration that immunization with synthetic antigens having clustered structures, without use of carrier proteins, mimics immunizations with cells or natural antigens. Future results in this area will be forthcoming.

13. Future Directions

It has been known for some time that specific types of glycolipids or glycoproteins, which may be detectable in normal cells by immunohistology, are more highly expressed in tumors. Furthermore, high levels of expression on tumor cells causes an antibody response, consequently rendering the cell-surface glycoconjugate recognizable as a tumor-associated antigen. The idea of such glycoconjugates as tumor-associated antigens is the basis for using carbohydrates in the development of antitumor vaccines.

Cancer carbohydrate antigens such as TF, Tn, KH-1, Le^y, and Globo-H are suitable targets for both active and passive immunotherapies because they have been carefully characterized as being over-expressed at the surface of malignant cells in a variety of cancers. In addition, they have been immunocharacterized by suitable monoclonal antibodies and therefore have relevant serological markers available for immunological studies. We have conducted such studies with the hope that patients immunized in an adjuvant setting with synthetic carbohydrate vaccines would produce antibodies reactive with cancer cells and that the production of such antibodies would mitigate against tumor spread so enabling a more favorable prognosis.

With respect to the mucin-like constructs and the cassette strategy, while complexities will undoubtedly be encountered on a case-to-case basis, we believe that the results shown here constitute validation and broad demonstration that the required chemistry can be achieved in the general case. The synthesis of **119** is a particularly striking example in which we have achieved success. In fact, we are in the process of applying the cassette methodology to the synthesis of antigenic structures containing the Globo-H epitope. Increasingly sophisticated and, we hope, increasingly realistic cell-surface molecular mimics can now be assembled and evaluated, both in regard to spectroscopy[104] and immune recognition.

In conclusion, it is clear that chemical synthesis has met the challenge of complex glycoconjugate synthesis and will continue to do so. The ability to probe intricate structural and, in due course, mechanistic questions with regard to anticancer vaccine development will, accordingly, grow. Although the studies which may be particularly important with regard to cancer vaccines are still in their infancy, the necessary complex materials required for further preclinical and clinical studies are becoming increasingly available. It is to be hoped that the creative interfacing of complex target-oriented synthesis and immunology will bring with it clinical benefits.

Abbreviations

Ac	acetyl
Bn	benzyl
Boc	*tert*-butoxycarbonyl
BSA	bovine serum albumin
Bz	benzoyl
CDC	complement-dependent cytotoxicity
CMPI	2-chloro-1-methylpyridinium iodide
coll	collidine (2,4,6-Trimethylpyridine)
Cp	cyclopentadienyl
DAST	diethylaminosulfurtrifluoride
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DIEA	diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMDO	3,3-dimethyldioxirane
DTBP	di-*tert*-butylpyridine
EDC	*N'*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide
ELISA	enzyme-linked immunosorbant assay
Fmoc	fluoren-9-ylmethoxycarbonyl
HATU	*N*-[(dimethylamino)-1*H*-1,2,3-triazole[4,5-*b*]-pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate
HOAt	7-aza-1-hydroxy-1*H*-benzotriazole
IIDQ	2-(2-methylpropoxy)-1(2*H*)-quinoline carboxylic acid-(2-methylpropyl)ester
KLH	keyhole limpet hemocyanin
Lev	levulinate (4-oxopentanoate)
LHMDS	lithium bis(trimethylsilyl)amide
mAb	monoclonal antibody
NHS	*N*-hydroxysuccinimide
NIS	*N*-iodosuccinimide
OTf	trifluormethanesulfonate
Pam_3Cys	tripalmitoyl-*S*-glycerylcysteinylserine
PMB	*para*-methoxybenzyl
Pn	pentenyl
PPTS	pyridinium-*p*-toluolsulfonate
PSA	prostate specific antigen
SAMA-(OPfp)	*S*-acetylmercaptoacetic acid pentafluorophenyl ester
SCLC	small cell lung cancer
TBAF	tetrabutylammoniumfluoride
TBDPS	*tert*-butyldiphenylsilyl
TBS	*tert*-butyldimethylsilyl
TEA	triethylamine
TES	triethylsilyl
Tf	trifluormethanesulfonyl
TFA	trifluoroacetic acid
TFAA	trifluoroacetic anhydride
TIPS	triisopropylsilyl
TMS	trimethylsilyl

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Exhibit B

THE SOLUTION CONFORMATION OF THE Le^x GROUP

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The solution conformation of the non-reducing terminal Galβ1$\rightarrow$4(Fucα1$\rightarrow$3)GlcNAc (Lewis X or Le^x) group in the oligosaccharide Lacto-N-fucopentaose (LNFP) III has been determined by high resolution ^{1}H NMR spectroscopy and semi-empirical quantum mechanical calculations. The two methods give the same single conformer for the Le^x group showing close packing of the Gal and Fuc rings. The metal binding properties and homotypic oligomer formation of LNFP III have also been investigated by NMR spectroscopy. No evidence for metal binding or high-affinity homotypic oligomer formation has been found.

Cell surface carbohydrates are known to be involved in specific cell-cell interactions via carbohydrate-lectin complexes [1]. In particular, interest has focused on the family of oligosaccharides containing one or more non-reducing terminal Le^x groups (fig 1a) or substituted Le^x groups (especially sialyl-Le^x) [2,3]. Differential expression of cell surface Le^x and sialyl-Le^x determinants has also been observed during different stages of cellular development, differentiation and maturation [4].

More recently, carbohydrates, and particularly oligosaccharides containing the non-reducing terminal Le^x group, have been implicated in non-specific cellular adhesion interactions [5 and refs therein]. It has been proposed [5] that this type of cell-cell recognition is based on divalent metal ion (calcium, magnesium, manganese) dependent, homotypic carbohydrate-carbohydrate interactions, between Le^x groups.

The solution structure, the ability to form homo-oligomers and the metal binding properties of the Le^x group are thus of considerable current interest, due to the role of Le^x as a possible ligand for cell surface receptors. There is also interest in its possible role in non-specific cell-cell interactions and as the core structure for other members of the Le^x family.

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```
a  Galβ1
        ↘
         4
          GlcNAc                    Leˣ
         3
        ↗
   Fucα1

b  Galβ1
   (2)  ↘
         4
          GlcNAcβ1→3 Galβ1→4Glc     LNFP III
         3            (1)
        ↗
   Fucα1
```

Figure 1 . Oligosaccharides used in this study. (a) Leˣ oligosaccharide used for quantum mechanical calculations. (b) LNFP III oligosaccharide used for NMR studies.

MATERIALS AND METHODS

The oligosaccharide LNFP III (fig 1b) [6] was obtained as a lyophilised solid from Oxford Glycosystems Ltd. The initial sample was free of both calcium and magnesium, as determined by atomic absorption spectroscopy.

Samples were prepared and one- and two-dimensional spectra acquired as previously reported [7]. Mixing times of 80ms for RELAY spectra, 100 ms for TOCSY spectra, 400 ms with a random variation of ±20 ms for NOESY spectra and 60 ms, 80 ms and 120 ms for ROESY spectra were used. All spectra were recorded at 600 MHz and a temperature of 300 K and 1D and ROESY spectra were also recorded at 280 K. Chemical shifts were referenced to an internal standard of 3-(trimethyl-silyl)propionic acid (0.0 ppm).

Metal ion titrations were performed by adding small aliquots of concentrated solutions of either $CaCl_2$ (diamagnetic probe) or $MnCl_2$ (paramagnetic relaxation probe [8]) in ^2H_2O, pH = 6.0, to a 1.42 mM solution of the oligosaccharide in the NMR tube. Ca^{2+} titrations were carried out from Ca^{2+} : oligosaccharide = 1 : 30 to Ca^{2+} : oligosaccharide = 16 : 1 and Mn^{2+} titrations carried out from Mn^{2+} : oligosaccharide = 1 : 1500 to Mn^{2+} : oligosaccharide = 1 : 4.5.

The theoretical minimum energy conformation of the Le^x group was calculated by constructing the disaccharides Fucα1→3GlcNAc and Galβ1→4GlcNAc using the INSIGHT program (Biosym Technologies Inc.). The torsion angles $\phi = H1-C1-O1-CX$ and $\psi = C1-O1-CX-HX$ (where X = 3 or 4 for the two disaccharides respectively) were varied through the ranges $-210° < \phi, \psi \leq +210°$ in 30° steps. For each ϕ, ψ point, a minimum was calculated using the semi-empirical quantum mechanical program AMPAC [9] using the AM1 hamiltonian [10], allowing all other bond lengths and angles to relax fully. The calculations were performed twice in different ways. The first time ψ was varied in 30° steps at a fixed value of ϕ and the process then repeated incrementing ϕ by 30° each time. The second time ϕ was varied at fixed ψ and the process repeated incrementing ψ each time. At each point on the energy surface, the lowest of the two values for the energy was taken. The Le^x trisaccharide was constructed by using the values of ϕ and ψ giving the lowest energy for each separate linkage. The resultant trisaccharide structure was then further minimised using the AM1 hamiltonian and the keyword 'PRECISE' used in the AMPAC program.

RESULTS AND DISCUSSION

Solution conformation of LNFP III from NMR : The 1D ^1H NMR spectrum of LNFP III is shown in figure 2a. From immediate inspection of the 1D spectrum,

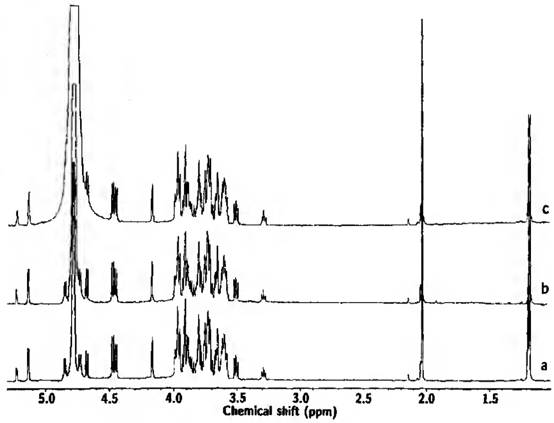

Figure 2 . 1D ^1H NMR spectra of LNFP III in ^2H_2O, 1.42 mM, pH = 6.0, recorded at 600 MHz and at a temperature of 300 K. (a) Absence of divalent metal ions. (b) Presence of 22.6 mM $CaCl_2$. (c) Presence of 0.05 mM $MnCl_2$.

comparison of chemical shifts with values from previously reported compounds [11,12] and COSY, RELAY and TOCSY (anomeric/ring region shown in fig 3a) spectra it is possible to assign all the resonances of the Fuc, the C1H to C4H proton resonances of both Gals, and most of the resonances of the Glc and GlcNAc (although there is some ambiguity as to the precise assignments). The sequence specific assignments for the two Gals were obtained from the cross-linkage NOE between GlcNAc C1H and Gal 1 C3H. These assignments, together with coupling constants derived from the 1D spectrum, are given in Table 1. The 1D spectrum at 280 K (not shown) is virtually identical to that at 300 K, except that the GlcNAc C1H resonance shifts upfield slightly and broadens.

The NOESY spectrum of LNFP III shows very weak intra-residue NOEs and only one inter-residue NOE (between GlcNAc C1H and Gal 1 C3H), indicating a correlation time of approximately $\frac{1}{\omega_o}$ (reciprocal of the Larmor precession frequency). Thus, ROESY experiments had to be used to obtain conformational information. As well as intra-residue ROEs, a number of inter-residue ROEs are also observed (anomeric/ring region shown in fig 3b). The conformationally important ROEs are listed in Table 2. Several inter-residue ROEs are also observed from the Gal 2 C1H resonance but these cannot be assigned unambiguously as the GlcNAc ring protons have not been fully assigned. An identical pattern of ROEs is observed at 280 K.

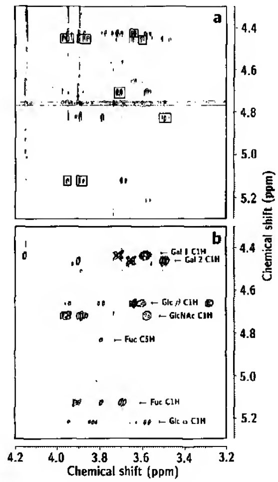

Figure 3. 2D ^1H NMR spectra of LNFP III in ^2H_2O, pH = 6.0, recorded at 600 MHz and at a temperature of 300 K. (a) Anomeric/ring region of the ROESY spectrum, mixing time = 80 ms. The inter-residue ROEs are boxed. (b) Anomeric/ring region of the TOCSY spectrum, mixing time = 100 ms. The labels show the resonance assignments in the F1 frequency domain.

The observation of ROEs other than those across a glycosidic linkage (from Gal 2 to Fuc) is highly unusual. A much more typical pattern of ROEs is observed for the rest of the molecule.

Table 1. ^1H NMR assignments for LNFP III oligosaccharide

Residue		Proton C1H	C2H	C3H	C4H	C5H	C6Hs	$COCH_3$
Glc α	δ ppm	5.22	3.58	3.84	(3.64)	(3.95)		–
	$J_{i,i+1}$ Hz	3.7					–	–
Glc β	δ ppm	4.66	3.29	3.64	3.61	(3.80)	(3.95)	–
	$J_{i,i+1}$ Hz	7.9	8.5				–	–
Gal 1	δ ppm	4.44	3.59	3.72	4.17	{3.80}		–
	$J_{i,i+1}$ Hz	7.8		3.2	<1.0		–	–
GlcNAc	δ ppm	4.72	3.97	3.89		(3.58)		2.02
	$J_{i,i+1}$ Hz	8.4					–	–
Fuc	δ ppm	5.13	3.70	3.92	3.80	4.84	1.18	–
	$J_{i,i+1}$ Hz	4.0	>8.0		<1.0	6.6	–	–
Gal 2	δ ppm	4.47	3.50	3.66	3.91			–
	$J_{i,i+1}$ Hz	7.8	9.75				–	–

Temperature = 300 K. Numbering sequence is given in figure 1. Assignments in brackets (..) are definite to residue but tentative to specific protons. Assignments in curly brackets {..} are tentative to residue.

Table 2. Assigned inter-residue ROEs observed for the LNFP III oligosaccharide

ROE			Strength
Gal 1 C1H	$\longleftrightarrow$	Glc β C3H	medium
Gal 1 C1H	$\longleftrightarrow$	Glc β C4H	partially obscured
GlcNAc C1H	$\longleftrightarrow$	Gal 1 C3H	medium
GlcNAc C1H	$\longleftrightarrow$	Gal 1 C4H	very weak
Fuc C1H	$\longleftrightarrow$	GlcNAc C2H	medium-weak
Fuc C1H	$\longleftrightarrow$	GlcNAc C3H	partially obscured
Fuc C1H	$\longleftrightarrow$	GlcNAc $COCH_3$	medium-weak
Fuc C5H	$\longleftrightarrow$	Gal 2 C2H	medium
Fuc C6H	$\longleftrightarrow$	Gal 2 C2H	medium-strong

Temperature = 300 K. Numbering sequence is given in figure 1.

In order to determine the solution conformation of the Le^x group, it is necessary to determine four torsion angles (ϕ and ψ for each linkage). Very loose ROE distance constraints were used because of the difficulty in extracting accurate distance information from ROE intensities [13]. The pattern of ROEs between Fuc C1H and the GlcNAc ring (analysed using the procedure of Wooten et al. [14]) is only consistent with a small range of ϕ, ψ torsion angles for the Fucα1$\rightarrow$3GlcNAc linkage ($\phi, \psi = 10\pm30°, 40\pm20°$). The Gal$\beta$1$\rightarrow$4GlcNAc linkage ($\phi, \psi \approx 30°, 30°$) is then defined by ROEs between the Gal and the Fuc and by steric interactions between these two residues.

Solution conformation of Le^x from quantum mechanical calculation : The potential energy surfaces for the Fucα1$\rightarrow$3GlcNAc and Galβ1$\rightarrow$4GlcNAc linkages are shown in figure 4. For the Fucα1$\rightarrow$3GlcNAc linkage, only one main minimum is observed, for the Galβ1$\rightarrow$4GlcNAc linkage, two main minima are seen. The energy minimised conformation of the Le^x group, based on the lowest energy minimum for each linkage, is shown in figure 5. The second conformation of Le^x that can be constructed using the minimum for the Galβ1$\rightarrow$4GlcNAc linkage at $\phi, \psi = 60°, -140°$ resulted in a very much higher energy, due to the highly unfavourable steric interactions between the Fuc and Gal rings. The potential energy surfaces for the component linkages indicate that the conformation of the Le^x must be relatively rigid, although molecular dynamics simulations are necessary to confirm this.

Metal ion binding to LNFP III studies : Figure 2 shows the 1D ^{1}H NMR spectrum of a 1.42 mM solution of LNFP III in the absence of divalent metal ions (fig 2a), in the presence of 22.6 mM Ca^{2+} (fig 2b) and in the presence of 0.14 mM Mn^{2+} (fig 2c). No detectable changes in the 1D spectrum are seen on the addition of Ca^{2+} to 22.6 mM. General broadening of resonances is seen on addition of Mn^{2+} to 0.3 mM but no specific

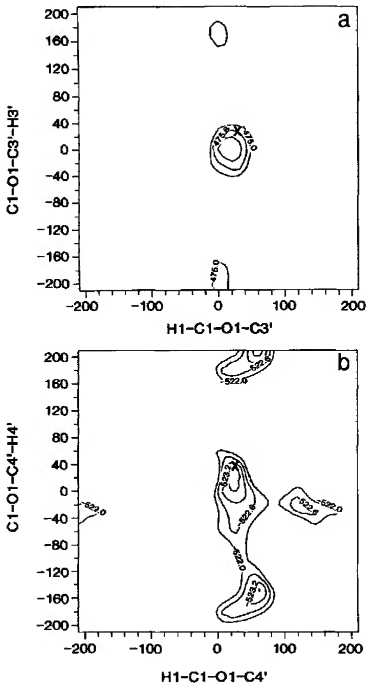

Figure 4 . Potential energy surfaces for (a) the Fucα1→3GlcNAc linkage and (b) the Galβ1→4GlcNAc linkage. Contour levels are in kcal/mol. X – linkage conformation in the energy minimised trisaccharide.

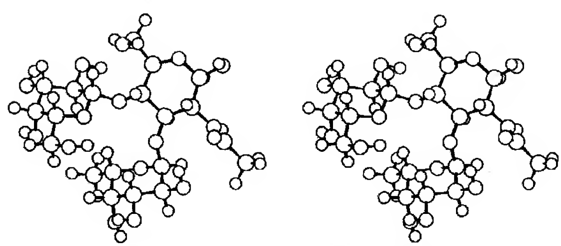

Figure 5 . Stereo diagram of the solution conformation of Le[x] determined from semi-empirical quantum-mechanical calculations and fully consistent with the NMR data. [Fuc-GlcNAc linkage, H1-C1-O1-C3 = 33.2° C1-O1-C3-H3 = 21.9°: Gal-GlcNAc linkage, H1-C1-O1-C4 = 27.1° C1-O1-C4-H4 = 33.4°].

effects are observed and the effects on the oligosaccharide resonances are several orders of magnitude less than the effect observed on the H^2HO resonance. Thus, there appear to be no specific or general interactions between LNFP III and Ca^{2+} or Mn^{2+}.

CONCLUSIONS

The solution conformation of the Le^x functional group has been independently determined by solution NMR and theoretical calculations. The agreement between these two methods is excellent, both giving the same single conformer. This provides experimental validation of the general theoretical approach used for the determination of the oligosaccharide conformation.

This structure is unusual in that there is very close packing ('stacking') of the fucose and galactose rings. Both the experimental and theoretical results are in good agreement with the results of Bush and co-workers [15,16], who examined the conformation and dynamics of the blood group trisaccharide derivative Fucα1$\rightarrow$2(GalNAcα1$\rightarrow$3)Galβ-O-methyl and found a single conformer over a wide range of temperature and solvent conditions due to the linkage stereochemistry, but are in contrast to the more elongated structures normally observed in small oligosaccharides [17].

The interactions between the Gal and Fuc ring appear to be chiefly hydrophobic and steric in nature, whilst a hydrogen bond can be formed between the Fuc C2OH and the GlcNAc CO. This provides a very rigid structure (leading to a minimum in loss of entropy on binding to a receptor) with a well-defined hydrophilic surface. It is also likely that the conformation of the Le^x determinant is not altered by further substitution, thus providing a common core structure for all other members of the Le^x family.

The Le^x group does not interact specifically with divalent metal cations at concentrations up to 22 mM and there is no evidence for Le^x homotypic oligomer formation in the presence or absence of divalent metal ions. Low affinity homotypic interactions between Le^x groups would be difficult to detect in aqueous solution but could still be responsible for bulk interactions between cells involving many surface groups.

ACKNOWLEDGMENT

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Exhibit C

JOC Article

Linear Synthesis of the Tumor-Associated Carbohydrate Antigens Globo-H, SSEA-3, and Gb3

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The tumor-associated carbohydrate antigens Globo-H, SSEA-3, and Gb3 were synthesized in a linear fashion using glycosyl phosphate monosaccharide building blocks. All of the building blocks were prepared from readily available common precursors. The difficult α-(1→4-*cis*)-galactosidic linkage was installed using a galactosyl phosphate donor with high selectivity. Introduction of the β-galactosamine unit required the screening a variety of amine protecting groups to ensure good donor reactivity and protecting group compatibility. An *N*-trichloroacetyl-protected galactosamine donor performed best for the installation of the β-glycosidic linkage. Conversion of the trichloroacetyl group to the *N*-acetyl group was achieved under mild conditions, fully compatible with the presence of multiple glycosidic bonds. This synthetic strategy is expected to be amenable to the synthesis of the globo-series of tumor antigens on solid-support.

Introduction

Altered glycosylation is a universal feature of cancer cells, and certain carbohydrate structures are markers of many tumors.[1,2] Like normal cells during embryonic development, tumor cells adhere to a variety of cell types, invade tissues, and undergo activation and rapid growth. Since changes in cellular glycosylation profiles are common during embryogenesis, it is not surprising that altered glycosylation is also characteristic of malignant transformation and tumor activation. A variety of changes occur in malignant cells, such as the loss of expression or excessive expression of certain structures, the persistence of incomplete or truncated structures, and the appearance of novel structures.[3] Carbohydrates are displayed on the surface of both normal and tumor cells in the context of membrane glycosphingolipids (GSLs) and glycoproteins. The carbohydrate epitopes of GSLs have been analyzed following extraction from whole cells and have been extensively studied for the treatment of cancer by both passive and active immunotherapy.[1,2]

One major class of GSLs is the globo-series of tumor antigens. Some prominent members of this family include Globo-H, Gb5, and Gb3 (Figure 1). Globo-H was first isolated and identified as an antigen on breast cancer cells[4,5] and is also expressed in prostate[6] and ovarian cancer.[7] A synthetic Globo-H construct is currently being evaluated and has shown very promising results as an antitumor vaccine in clinical trials.[8-10] Gb5 is abundant in testicular cancer[11-13] and is present during embryonic development.[14] In recognition of this latter property, the pentasaccharide moiety of Gb5 is often referred to as the stage specific embryonic antigen-3 (SSEA-3). Finally, Gb3 is highly enriched in ovarian cancer and Burkitt's B-cell lymphoma.[15]

Due to their importance as tumor markers and potential anticancer vaccines, several members of the globo-series of GSLs have been targets of total syntheses. Globo-H was first synthesized by Danishefsky and coworkers using the glycal assembly approach.[16,17] Further

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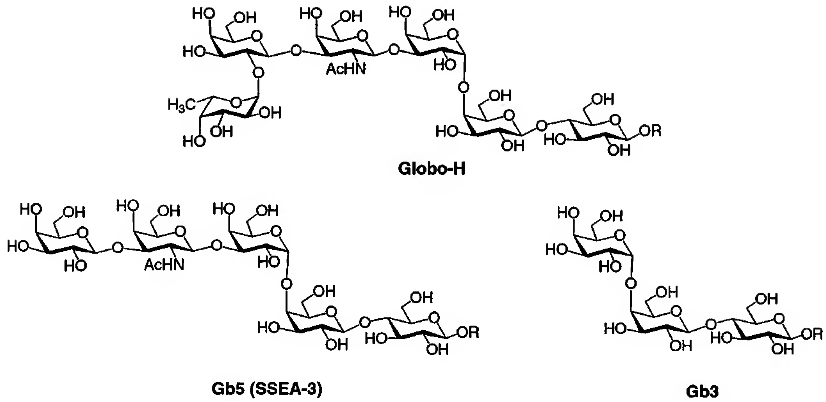

FIGURE 1. Globo-series of tumor-associated antigens (R = ceramide).

syntheses used trichloroacetimidate building blocks[18] and a two-directional glycosylation strategy.[19] Most recently, Wong and co-workers prepared Globo-H via a one-pot strategy using the computer program OptiMer to aid in synthesis planning.[20] The pentasaccharide SSEA-3 has been synthesized by Ogawa,[21] Danishefsky,[22] and Magnusson,[23] while the assembly of Gb3 has been reported by several groups.[24]

In this paper, we describe a solution-phase synthesis of the tumor antigens Globo-H, SSEA-3, and Gb3. A strictly linear strategy for the assembly of these oligosaccharides was investigated in anticipation of automating the synthesis on solid support.[25] Given their compatibility with solid-phase oligosaccharide chemistry, glycosyl phosphates were employed as donors in our solution-phase studies.[26,27] Glycosyl phosphates can be readily prepared from glycals using a one-pot procedure,[27] as well as from glycosyl trichloroacetimidates.[28] The use of glycosyl phosphate building blocks enabled the successful installation of the difficult α-galactosidic linkage, as well as the β-galactosamine moiety, in good yield with high stereoselectivity. The strategy described here is expected to be amenable to the preparation of the target tumor antigens on solid-support.

Results and Discussion

In keeping with our goal of automating solution-phase protocols on solid-support, six glycosyl donors were selected as building blocks for a sequential assembly of the Globo-H hexasaccharide **1** (Scheme 1). SSEA-3 and Gb3 became accessible by deprotection of the corresponding synthetic intermediates. This linear strategy differs markedly from previous routes, which had been striving for highest convergence. Acetyl and levulinoyl esters were chosen as temporary hydroxyl protecting groups, given their ease of removal[29] and compatibility with solid-phase synthesis.[25,30] The *n*-pentenyl group was selected for protection of the reducing end sugar in order to mimic the linker used during automated solid-phase synthesis.[25,31] In addition, the *n*-pentenyl glycoside can serve as a leaving group in a late-stage glycosylation[32] or as a handle for bioconjugation.[33]

Synthesis of the Lactose Disaccharide. The construction of the target oligosaccharides began with the preparation of the reducing end lactose disaccharide. Two monosaccharide building blocks had to be synthesized. Glucosyl phosphate **2** (Scheme 2) and galactosyl phosphate **3** were prepared using a one-pot procedure starting from 3,6-di-*O*-benzyl glucal **8**[29] and 3,6-di-*O*-benzyl galactal **10**,[34] respectively. It should be emphasized that while treatment of galactal **9** with benzyl bromide and sodium hydride at 0 °C[34] produced **10** in low yield (20%),

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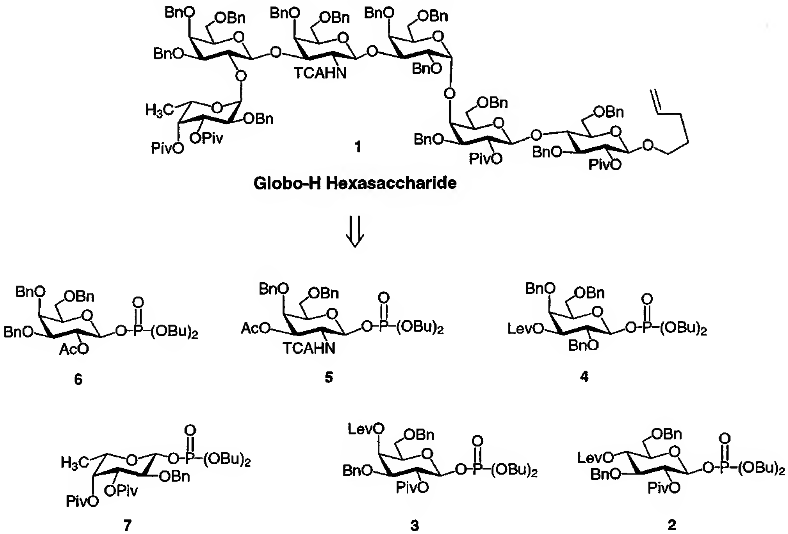
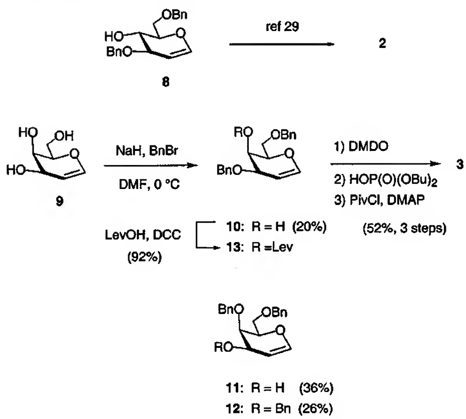
SCHEME 1. Retrosynthesis of the Protected Globo-H Hexasaccharide



SCHEME 2. Synthesis of Building Blocks 2 and 3



two other important intermediates, 4,6-di-*O*-benzyl galactal **11** (36%) and tri-*O*-benzyl galactal **12** (26%), were also generated in this reaction and were easily separated by column chromatography. Therefore, this procedure served as a starting point for the preparation of all four galactosyl building blocks (**3**–**6**) used in this synthesis. Protection of galactal **10** as the levulinate ester **13** (92%), followed by subsequent conversion to the glycosyl phosphate and esterification, produced **3** in 52% yield.

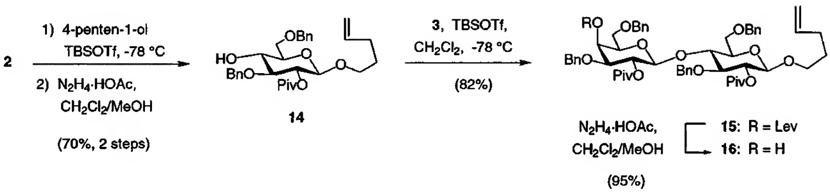
The assembly of the lactose disaccharide commenced with the installation of the *n*-pentenyl glycoside, followed by removal of the levulinoyl ester as described previously (Scheme 3).[29] Union of phosphate **3** with pentenyl glycoside **14** afforded lactose disaccharide **15** (82%), which upon deprotection with hydrazine yielded 95% of acceptor **16**.

Installation of the α-Galactosidic Linkage. A major challenge in the synthesis of the globo-series of tumor antigens is the installation of the α-(1→4-*cis*)-galactosidic linkage of the reducing end trisaccharide. While α-galactosidic linkages have been formed using a variety of glycosylating agents, the stereochemical outcome of this coupling is difficult to predict and is highly dependent on the glycosyl acceptor. High selectivity for all glycosylation reactions is mandatory, since this strategy is being developed for use on solid-support and cannot take advantage of the purification of reaction intermediates. To install the difficult α-linkage, we chose to explore the use of glycosyl phosphate **4**, given its compatibility with solid-phase oligosaccharide synthesis.

Since the presence of a benzyl ether at C2 was required to favor α-selectivity, donor **4** could not be synthesized directly from a glycal using the standard one-pot procedure[27,35] but was generated from a glycosyl trichloroacetimidate.[28] Hence, 4,6-di-*O*-benzyl galactal **11** was protected as the *p*-methoxybenzyl (PMB) ether **17**[17] (97%) and then converted to the allyl glycoside **18** (α:β = 1:5, 79%) by epoxidation with DMDO and solvolysis with allyl alcohol (Scheme 4). Installation of the C2 benzyl group **19** (71%), followed by exchange of the PMB ether for a levulinoyl ester, furnished **20** in 93% yield. Palladium-mediated cleavage of the allyl glycoside afforded the intermediate lactol, which was immediately converted to trichloroacetimidate **21** (76%, two steps) by reaction with trichloroacetonitrile and DBU. Transformation of the α-trichloroacetimidate (**21**) to the β-phosphate (**4**) was achieved in 90% yield upon exposure to dibutyl phos-

(35) Treatment of the intermediate glycosyl phosphate with sodium hydride and benzyl bromide leads to migration of the phosphate to yield the C2 phosphoryl benzyl glycoside.

SCHEME 3. Synthesis of the Lactose Disaccharide



SCHEME 4. Synthesis of the C2 Benzyl-Protected Galactose Donor

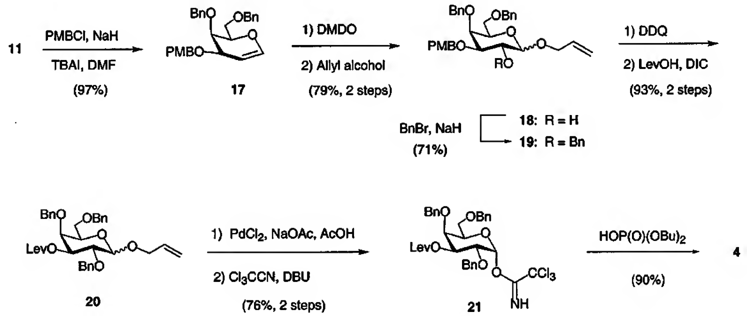
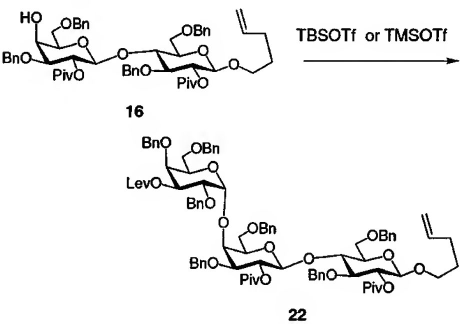
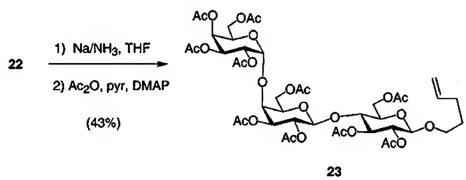


TABLE 1. Coupling Conditions Used To Generate the Gb3 Trisaccharide



donor	solvent	temp (°C)	α:β selectivity	yield (%)
4	CH_2Cl_2	−78	11:1	58
4	CH_2Cl_2/THF	−20	1:1	80
4	CH_2Cl_2/THF	−78	20:1	61
4	CH_2Cl_2/Et_2O	−78	20:1	79
21	CH_2Cl_2	0	α only	54

SCHEME 5. Deprotection of the Gb3 Trisaccharide



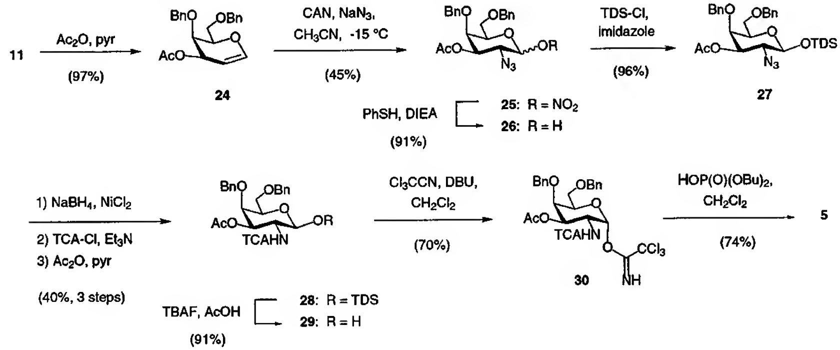
phate. It should be noted that attempts to generate the glycosyl phosphate directly from the lactol led to the formation of 1,1 coupled disaccharides.

Synthesis of the Gb3 Trisaccharide. With the desired glycosyl phosphate in hand, conditions for glycosylation of the hindered lactose acceptor **16** were explored (Table 1). Initial reaction conditions employing TBSOTf as a promoter in dichloromethane at −78 °C led to the formation of target trisaccharide **22** in 58% yield but with only moderate α-selectivity (α:β = 11:1). Both the yield and stereoselectivity of the reaction improved when a mixture of ether/dichloromethane was used (79%, α:β = 20:1). Reaction temperature proved crucial for achieving high α-selectivity. When the reaction was carried out at −20 °C, a 1:1 mixture of α- and β-anomers was obtained. The intermediate trichloroacetimidate **21** also proved to be a suitable glycosylating agent for the preparation of trisaccharide **22**. Reaction of **21** with disaccharide **16** at 0 °C in dichloromethane yielded α-linked trisaccharide **22** in 54% yield.

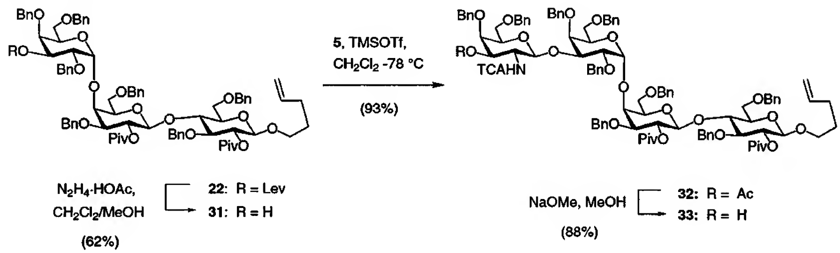
Removal of all ester and benzyl ether protecting groups of trisaccharide **22** was accomplished by a dissolving-metal reduction (Scheme 5). Subsequent treatment with acetic anhydride in pyridine furnished the peracetylated Gb3 trisaccharide **23** in 43%.

Synthesis of the Galactosamine Building Block. Having installed the challenging α-galactosidic linkage, we turned our attention to the synthesis of a suitable glycosylating agent for the introduction of the galactosamine unit. An important consideration in designing the galactosamine building block was the selection of an amine protecting group. A number of strategies for masking the C2 amino group were investigated, including protection as the *N*-carbamate (Troc and Cbz), *N*-tri-

SCHEME 6. Synthesis of *N*-TCA-Protected Galactosamine Donor 5



SCHEME 7. Installation of *N*-TCA-Protected Galactosamine



chloroacetamide, *N*-phthalimide, and azide. Glycosyl phosphate **5** bearing a C2 *N*-trichloroacetamide performed best. The *N*-trichloroacetyl (TCA) group has been reported to ensure high β-selectivity in glycosylation reactions, can be converted directly into the corresponding *N*-acetyl group under mild conditions that are fully compatible with sensitive glycosidic bonds, and has been used in the synthesis of several complex oligosaccharides.[36–39]

The synthesis of the required galactosamine donor commenced with 4,6-di-*O*-benzyl galactal **11**, which was protected as the C3 acetate **24**[40] and then subjected to an azidonitration reaction (Scheme 6). The resulting product (**25**) was hydrolyzed by exposure to thiophenol in the presence of diisopropylethylamine (DIEA), and lactol **26** was protected as the anomeric silyl ether (**27**) using thexyldimethylsilyl (TDS) chloride (96%). Reduction of the azido group with sodium borohydride in ethanol afforded the C2 amine, which was then treated with trichloroacetyl (TCA) chloride in the presence of triethylamine. Due to partial de-*O*-acetylation during the reaction with sodium borohydride, the crude mixture was

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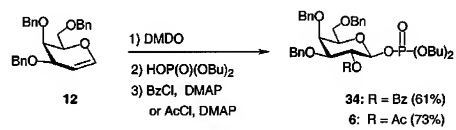
SCHEME 8. Synthesis of C2 Ester-Protected Galactose Donors 6 and 34

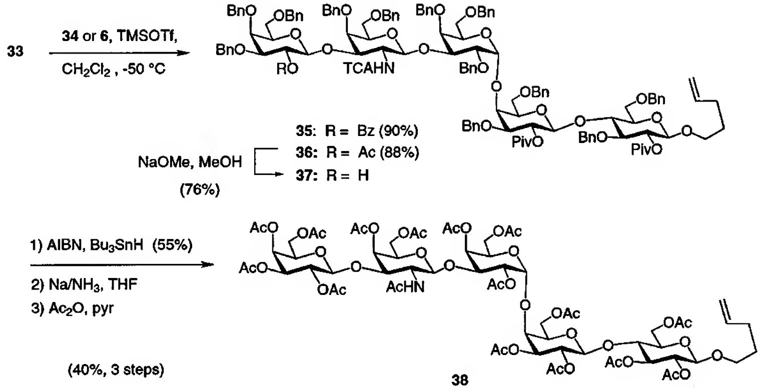


reacetylated with acetic anhydride in pyridine to procure *N*-TCA protected **28** in 40% yield. Removal of the anomeric TDS group under the agency of TBAF (91%) followed by reaction of lactol **29** with trichloroacetonitrile in the presence of DBU afforded trichloroacetimidate **30** in 70% yield. Conversion of **30** into glycosyl phosphate **5** was accomplished in good yield (74%) by reaction with dibutyl phosphate.

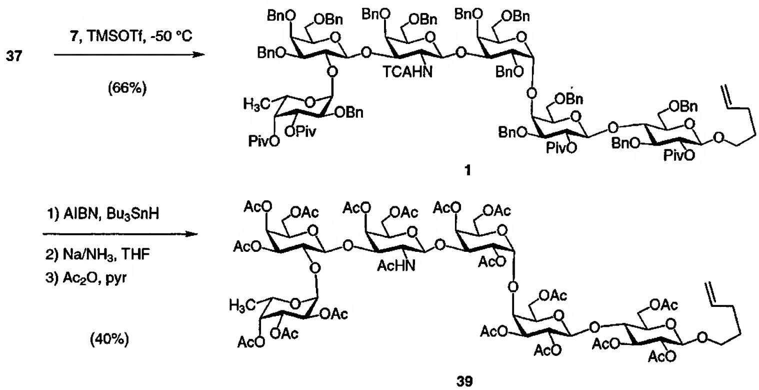
Reaction of trisaccharide **22** with hydrazine acetate provided acceptor **31** in 62% yield (Scheme 7). Glycosylation of **31** with **5** at -78 °C in the presence of TMSOTf proceeded in excellent yield (93%) to afford exclusively the desired β-linked product **32**. In comparison, attempts to glycosylate **31** with trichloroacetimidate donor **30** using a catalytic amount of TMSOTf resulted only in acceptor decomposition. Deacetylation of tetrasaccharide **32** with sodium methoxide provided acceptor **33** in 88% yield.

Synthesis of the SSEA-3 Pentasaccharide. Elaboration of tetrasaccharide **33** to the SSEA-3 precursor required the introduction of a galactose unit at the C3 position of the terminal galactosamine residue. To achieve

SCHEME 9. Completion of the SSEA-3 Pentasaccharide



SCHEME 10. Completion of the Globo-H Hexasaccharide



high β-selectivity in the glycosylation reaction, we investigated the use of a galactose building block bearing a C2 ester. Initially, a benzoate group was chosen as a means of protection for the C2 hydroxyl group. Building block **34**[27] was synthesized in one-pot starting from tri-*O*-benzyl galactal **12** (Scheme 8). While the union of phosphate **34** with tetrasaccharide **33** proceeded smoothly (90% yield) (Scheme 9), subsequent deprotection of the C2 benzoyl ester (**35**) proved problematic. Complete removal of the benzoate could not be achieved without concomitant loss of the pivaloyl esters. Replacement of the C2 benzoyl group of donor **34** with an acetate (**6**) enabled the successful synthesis of the target pentasaccharide. Glycosylation of tetrasaccharide **33** with phosphate donor **6** under the agency of TMSOTf provided pentasaccharide **36** in good yield (88%). Deacetylation of **36** with sodium methoxide cleanly afforded compound **37** (76%).

Following conversion of the *N*-TCA group to the corresponding *N*-acetamide with tributylstannane and AIBN, the protected pentasaccharide was converted to peracetylated SSEA-3 **38** (40%) by a dissolving-metal reduction and reaction with acetic anhydride. It is important to note that prior conversion of the *N*-TCA-group to the *N*-acetamide was crucial for the success of this reaction sequence. Subjection of *N*-TCA-protected pentasaccharide **37** to dissolving-metal reduction led to cleavage of the β-galactosamine linkage, producing trisaccharide **23** after peracetylation.

Completion of the Globo-H Hexasaccharide. Fucosyl phosphate **7** had performed well previously for the installation of α-(1→2-*cis*) glycosidic linkages[29] and was employed for the completion of the target hexasaccharide. Glycosylation of pentasaccharide **37** with fucosyl phosphate **7** provided protected Globo-H hexasaccharide **1** in 66% yield with complete α-selectivity (Scheme 10). Deprotection of **1** via dissolving-metal reduction as above provided peracetylated Globo-H **39** in 40% yield. NMR and mass spectral analysis of **39** was in accordance with previously reported data.[33]

Conclusions

In summary, the synthesis of three members of the globo-series of tumor antigens was accomplished in a

linear fashion using six glycosyl phosphate building blocks. In the context of these syntheses, methods for the installation of α-galactosidic and β-galactosamine linkages using glycosyl phosphates were developed. The strategy devised for this solution-phase synthesis is currently being applied to the synthesis of the globo-series of tumor antigens on solid-support in a fully automated fashion.

Experimental Section

General Methods. All chemicals were reagent grade and used as supplied, unless otherwise noted. Dichloromethane (CH_2Cl_2), tetrahydrofuran (THF), diethyl ether (Et_2O), and toluene were purified by a JT Baker Cycle-Trainer Solvent Delivery System. Analytical thin-layer chromatography was performed on silica gel 60 F_{254} plates (0.25 mm). Compounds were visualized by dipping the plates in a cerium sulfate-ammonium molybdate solution followed by heating. Flash chromatography was performed using forced flow of the indicated solvent on Silicylce silica (230−400 mesh). NMR spectra (^{1}H at 400 MHz, ^{13}C at 100 MHz) were recorded in $CDCl_3$ as the solvent and chemical shifts are reported in parts per million (δ) relative to $CHCl_3$ as an internal reference. ^{31}P spectra (120 MHz) are reported in δ relative to H_3PO_4 (0.0 ppm) as an external reference. Optical rotations were measured at 24 °C.

3,6-Di-*O*-benzyl-4-*O*-levulinoyl-D-galactal 13. Galactal **10**[34] (3.94 g, 10.7 mmol) was dissolved in CH_2Cl_2 (80 mL) and cooled to 0 °C. DCC (3.53 g, 17.1 mmol), DMAP (131 mg, 1.07 mmol), and levulinic acid (1.5 mL, 15.0 mmol) were added, and the mixture was stirred for 14 h at room temperature. The mixture was filtered, washed twice with water, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (3:1 hexane/EtOAc) to afford 4.20 g (92%) of **13** as a pale yellow oil. $[\alpha]_D$: +1.13 (c = 1.6, CH_2Cl_2). IR (thin film): 2920, 1738, 1717, 1362, 1155, 1099 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.28−7.18 (m, 10 H), 6.32 (dd, J = 6.3, 1.8 Hz, 1 H), 5.56−5.52 (m, 1 H), 4.68−4.65 (m, 1 H), 4.57 (d, J = 11.8 Hz, 1 H), 4.50 (d, J = 11.7 Hz, 1 H), 4.45 (d, J = 11.7 Hz, 1 H), 4.39 (d, J = 11.8 Hz, 1 H), 4.17−4.15 (m, 1 H), 4.12−4.08 (m, 1 H), 3.60 (dd, J = 9.8, 6.2 Hz, 1 H), 3.50 (dd, J = 9.8, 6.3 Hz, 1 H), 2.67−2.53 (m, 4 H), 2.07 (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.55, 172.47, 144.65, 138.18, 137.86, 128.62, 128.57, 128.26, 128.01, 127.87, 101.35, 74.56, 73.87, 71.15, 69.63, 68.61, 63.57, 38.17, 30.00, 28.29. ESI-MS: m/z (M + Na)$^+$ calcd 447.1778, obsd 447.1752.

Dibutyl 3,6-Di-*O*-benzyl-4-*O*-levulinoyl-2-*O*-pivaloyl-β-D-galactopyranoside Phosphate 3. Galactal **13** (0.831 g, 2.55 mmol) was dissolved in CH_2Cl_2 (15 mL) and cooled to 0 °C. DMDO (0.08 M in acetone, 45 mL, 3.6 mmol) was added and the reaction was stirred for 20 min. The solvent was evaporated at 0 °C and the residue dissolved in CH_2Cl_2 (40 mL) and cooled to −78 °C. A solution of dibutyl phosphate (0.56 mL, 2.8 mmol) in CH_2Cl_2 (10 mL) was added dropwise. After stirring for 10 min, the mixture was warmed to 0 °C, and DMAP (1.24 g, 10.2 mmol) and pivaloyl chloride (0.63 mL, 5.10 mmol) were added. After stirring for 14 h the solvent was removed in vacuo, and the residue was purified by flash chromatography (1:1 hexane/EtOAc), yielding 980 mg (52%) of **3** as a colorless oil. $[\alpha]_D$: +25.2 (c = 1.28, CH_2Cl_2). IR (thin film): 2928, 1740, 1494, 1277, 1050 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37−7.23 (m, 10 H), 5.67−5.66 (m, 1 H), 5.23−5.21 (m, 2 H), 4.69 (d, J = 11.4 Hz, 1 H), 4.51−4.50 (m, 2 H), 4.38 (d, J = 11.4 Hz, 1 H), 4.06−3.98 (m, 4 H), 3.88−3.87 (m, 1 H), 3.65−3.54 (m, 3 H), 2.74−2.62 (m, 4 H), 2.15 (s, 3 H), 1.63−1.58 (m, 4 H), 1.40−1.32 (m, 4 H), 1.19 (s, 9 H), 0.93−0.87 (m, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.56, 177.17, 172.05, 137.77, 137.26, 128.62, 128.58, 128.50, 128.28, 128.23, 128.09, 128.05, 128.01, 96.97, 77.15, 73.10, 71.71, 70.31, 68.21, 68.12, 67.34, 66.09, 38.18, 32.28. 32.21, 30.00, 28.17, 27.33, 18.78, 18.75, 13.77, 13.74. ^{31}P NMR (120 MHz, $CDCl_3$): δ −2.12. ESI-MS: m/z (M + Na)$^+$ calcd 757.3323, obsd 757.3313.

***n*-Pentenyl 3,6-Di-*O*-benzyl-4-*O*-levulinoyl-2-*O*-pivaloyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*O*-pivaloyl-β-D-glucopyranoside 15.** Glycosyl phosphate **3** (1.83 g, 2.49 mmol) and monosaccharide **14**[29] (1.44 g, 2.81 mmol) were coevaporated three times with toluene, dissolved in CH_2Cl_2 (60 mL), and cooled to −78 °C. TBSOTf (0.83 mL, 3.61 mmol) was added and the mixture was warmed slowly to −60 °C. After 1 h Et_3N (5 mL) was added and the mixture was poured into a saturated solution of $NaHCO_3$ (100 mL). The aqueous phase was extracted twice with CH_2Cl_2, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (3:1 hexane/EtOAc) to afford 2.12 g (82%) of **15** as a colorless oil. $[\alpha]_D$: −1.03 (c = 1.28, CH_2Cl_2). IR (thin film): 3438, 2970, 2932, 2871, 1738, 1367, 1277 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38−7.20 (m, 20 H), 5.76−5.38 (m, 1 H), 5.59 (d, J = 3.0 Hz, 1 H), 5.10 (dd, J = 10.0, 8.1 Hz, 1 H), 4.95−5.04 (m, 4 H), 4.77 (d, J = 12.1 Hz, 1 H), 4.69 (d, J = 11.4 Hz, 1 H), 4.56 (d, J = 10.7 Hz, 1 H), 4.46−4.36 (m, 4 H), 4.32−4.29 (m, 2 H), 4.07 (app t, J = 9.3 Hz, 1 H), 3.89−3.83 (m, 1 H), 3.77−3.70 (m, 2 H), 3.65−3.60 (m, 1 H), 3.54−3.51 (m, 1 H), 3.47−3.42 (m, 1 H), 3.38−3.28 (m, 4 H), 2.73−2.49 (m, 4 H), 2.08 (s, 3 H), 2.13−2.06 (m, 2 H), 1.72−1.64 (m, 2 H), 1.18 (s, 9 H), 1.15 (s, 9 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.85, 176.97, 176.75, 172.11, 138.89, 138.26, 138.12, 138.02, 137.63, 128.73, 128.65, 128.57, 128.41, 128.36, 128.26, 128.19, 128.03, 127.89, 127.87, 127.81, 127.69, 127.47, 127.39, 75.39, 75.31, 74.62, 73.76, 72.39, 72.24, 71.41, 71.15, 69.07, 68.05, 67.44, 66.31, 38.93, 38.89, 38.15, 30.21, 29.87, 28.93, 28.10, 27.43, 27.33.

***n*-Pentenyl 3,6-Di-*O*-benzyl-2-*O*-pivaloyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*O*-pivaloyl-β-D-glucopyranoside 16.** Disaccharide **15** (1.98 g, 1.91 mmol) was dissolved in CH_2Cl_2 (100 mL). A solution of hydrazine acetate (193 mg, 2.10 mmol) in MeOH (10 mL) was added, and the mixture was stirred for 1 h at room temperature and concentrated. The residue was purified by flash chromatography (4:1 hexane/EtOAc), yielding 1.70 g (95%) of **16** as a colorless oil. $[\alpha]_D$: +13.5 (c = 0.80, CH_2Cl_2). IR (thin film): 2928, 1738, 1134, 1062 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39−7.19 (m, 20 H), 5.83−5.76 (m, 1 H), 5.20 (dd, J = 9.8, 8.1 Hz, 1 H), 5.05−4.95 (m, 4 H), 4.76 (d, J = 12.1 Hz, 1 H), 4.65 (d, J = 11.8 Hz, 1 H), 4.56 (d, J = 10.9 Hz, 1 H), 4.51 (d, J = 11.8 Hz, 1 H), 4.47−4.38 (m, 3 H), 4.36 (d, J = 8.0 Hz, 1 H), 4.31 (d, J = 11.9 Hz, 1 H), 4.05 (app t, J = 9.3 Hz, 1 H), 4.02−4.01 (m, 1 H), 3.89−3.83 (m, 1 H), 3.63 (app t, J = 9.1 Hz, 1 H), 3.57 (dd, J = 9.5, 6.6 Hz, 1 H), 3.47−3.31 (m, 5 H), 2.25 (br s, 1 H), 2.13−2.06 (m, 2 H), 1.68−1.64 (m, 4 H), 1.19 (s, 9 H), 1.18 (s, 9 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.08, 176.82, 139.00, 138.31, 138.29, 138.21, 137.50, 128.69, 128.63, 128.57, 128.24, 128.17, 127.95, 127.90, 127.88, 127.80, 127.69, 127.28, 114.99, 101.51, 99.79, 81.05, 79.42, 75.40, 75.35, 74.74, 73.76, 73.74, 73.35, 72.51, 71.57, 71.15, 69.09, 68.46, 68.18, 65.78, 38.98, 38.91, 30.24, 28.96, 27.47, 27.34. ESI-MS: m/z (M + Na)$^+$ calcd 961.4709, obsd 961.4725.

Allyl 4,6-Di-*O*-benzyl-3-*O*-*p*-methoxybenzyl-α/β-D-galactopyranoside 18. To a solution of di-*O*-benzyl galactal **11**[34] (1.26 g, 3.86 mmol) in DMF (20 mL) were added NaH (176 mg, 4.4 mmol), *p*-methoxybenzyl chloride (600 μL, 4.4 mmol), and TBAI (70 mg, 0.19 mmol) at 0 °C. The reaction was stirred for 16 h at room temperature, diluted with EtOAc, washed three times with water, and dried over $MgSO_4$. The organic phase was concentrated in vacuo and the crude residue was purified by flash chromatography (6:1 hexanes/EtOAc) to afford 1.65 g (97%) of **17**[17] as a colorless oil. Compound **17** (1.65 g, 3.70 mmol) was dissolved in CH_2Cl_2 (30 mL) and cooled to 0 °C. A solution of DMDO (0.08 M in acetone, 60 mL, 4.8 mmol) was added and the mixture was stirred for 10 min and then concentrated in vacuo at 0 °C. The resulting residue was dissolved in allyl alcohol (30 mL) and stirred at room temper-

ature for 16 h. The solution was concentrated and the crude product was purified by flash chromatography (3:1 hexanes/EtOAc) to afford 1.52 g (79%, α:β = 1:5) of **18** as a colorless oil. IR (thin film): 3460, 2914, 2869, 2107, 1612, 1513, 1454, 1248, 1078 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, β-anomer): δ 7.39−7.28 (m, 14 H), 6.91 (app d, J = 8.6 Hz, 2 H), 5.98−5.92 (m, 1 H), 5.32 (dd, J = 17.2, 1.4 Hz, 1 H), 5.21 (app d, J = 10.4 Hz, 1 H), 4.91 (d, J = 11.6 Hz, 1 H), 4.71−4.59 (m, 2 H), 4.52−4.44 (m, 2 H), 4.42−4.36 (m, 1 H), 4.32 (d, J = 7.7 Hz, 1 H), 4.16−4.12 (m, 1 H), 4.00−3.97 (m, 1 H), 3.93 (app d, J = 2.5 Hz, 1 H), 3.38 (s, 3 H), 3.66−3.59 (m, 2 H), 3.44 (dd, J = 9.8, 2.8 Hz, 1 H), 2.42 (br s, 1 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.75, 138.92, 138.26, 130.48, 129.29, 128.88, 128.69, 128.62, 128.34, 128.26, 128.23, 128.00, 118.25, 114.35, 114.30, 102.42, 82.05, 75.09, 74.38, 74.16, 73.99, 73.14, 72.45, 71.60, 70.39, 69.15, 55.71, 21.51, 14.64. ESI-MS: m/z (M + Na)$^+$ calcd 543.2359, obsd 543.2359.

Allyl 2,4,6-Tri-*O*-benzyl-3-*O*-*p*-methoxybenzyl-α/β-D-galactopyranoside 19. To a solution of **18** (1.5 g, 2.9 mmol) in DMF (20 mL) were added NaH (140 mg, 3.5 mmol) and benzyl bromide (420 μL, 3.5 mmol) at 0 °C. The mixture was stirred for 4 h and then quenched with water, diluted with EtOAc, and washed twice with water and once with brine. The organic phase was dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash chromatography (6:1 hexanes/EtOAc) to afford 1.60 g (71%, α:β = 1:5) of **19** as a white solid. IR (thin film): 2915, 2867, 2360, 2341, 1513, 1454, 1248, 1099, 1077 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, β-anomer): δ 7.43−7.28 (m, 19 H), 6.90 (d, J = 11.6 Hz, 1 H), 6.89 (d, J = 11.5 Hz, 1 H), 6.01−5.93 (m, 1 H), 5.35 (dd, J = 18.0, 2.0 Hz, 1 H), 5.20 (dd, J = 12.2, 1.4 Hz, 1 H), 4.97 (d, J = 11.7 Hz, 1 H), 4.96 (d, J = 10.8 Hz, 1 H), 4.80 (d, J = 10.8 Hz, 1 H), 4.47−4.42 (m, 3 H), 4.17−4.13 (m, 1 H), 3.90−3.86 (m, 1 H), 3.84 (s, 3 H), 3.61 (dd, J = 6.3, 3.0 Hz, 1 H), 3.57−3.52 (m, 2 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.56, 139.21, 139.08, 138.34, 134.63, 131.04, 129.64, 129.58, 128.86, 128.80, 128.71, 128.67, 128.59, 128.44, 128.32, 128.22, 128.16, 127.98, 127.95, 117.47, 103.38, 82.31, 80.02, 75.70, 74.86, 73.97, 73.84, 73.39, 73.15, 69.34, 68.63, 55.70. ESI-MS: m/z (M + Na)$^+$ calcd 633.2823, obsd 633.2828.

Allyl 2,4,6-Tri-*O*-benzyl-3-*O*-levulinoyl-α/β-D-galactopyranoside 20. To a solution of **19** (1.20 g, 1.96 mmol) in CH_2Cl_2 (20 mL) were added water (1.0 mL) and DDQ (556 mg, 2.45 mmol). The reaction was stirred for 1 h at room temperature and then poured into a saturated solution of $NaHCO_3$ and extracted three times with CH_2Cl_2. The organic phases were combined, dried over $MgSO_4$, filtered, and concentrated. The crude residue was dissolved in CH_2Cl_2 (20 mL), and levulinic acid (318 mg, 2.74 mmol), DIC (490 μL, 3.14 mmol), and DMAP (24 mg, 0.20 mmol) were added. The mixture was stirred for 16 h at room temperature and then diluted with CH_2Cl_2, washed with H_2O and saturated aqueous $NaHCO_3$, dried over $MgSO_4$, and filtered. The solvent was removed in vacuo and the residue was purified by flash chromatography (3:1 → 2:1 hexanes/EtOAc) to afford 1.07 g (93%, α:β = 1:5) of **20** as a colorless oil. IR (thin film): 2920, 1738, 1718, 1361, 1209, 1158, 1074 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, β-anomer): δ 7.38−7.28 (m, 15 H), 5.98−5.93 (m, 1 H), 5.35 (dd, J = 17.2, 1.6 Hz, 1 H), 5.20 (dd, J = 10.5, 1.4 Hz, 1 H), 4.97−4.91 (m, 2 H), 4.73−4.66 (m, 2 H), 4.57−4.42 (m, 5 H), 4.17−4.14 (m, 1 H), 3.96 (app d, J = 2.9 Hz, 1 H), 3.84 (dd, J = 10.2, 7.7 Hz, 1 H), 3.69−3.76 (m, 1 H), 3.62−3.55 (m, 2 H), 2.76−2.39 (m, 4 H), 2.15 (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.74, 172.64, 138.99, 138.70, 138.25, 134.39, 128.85, 128.77, 128.67, 128.63, 128.54, 128.46, 128.40, 128.31, 128.24, 128.20, 128.12, 128.02, 127.97, 117.63, 103.21, 77.30, 75.59, 75.34, 75.10, 74.82, 73.89, 73.79, 73.57, 70.63, 68.78, 38.19, 30.24, 28.38, 28.30. ESI-MS: m/z (M + Na)$^+$ calcd 611.2615, obsd 611.2619.

2,4,6-Tri-*O*-benzyl-3-*O*-levulinoyl-α-D-galactopyranosyl Trichloracetimidate 21. Compound **20** (906 mg, 1.54 mmol) was dissolved in AcOH (9 mL). Water (300 μL) was added, followed by NaOAc (290 mg, 3.54 mmol) and $PdCl_2$ (314 mg, 1.77 mmol), and the mixture was stirred for 16 h at room temperature. The reaction mixture was diluted with EtOAc and washed with water, saturated aqueous $NaHCO_3$, and brine. The organic phase was dried over $MgSO_4$, filtered, and concentrated to give the desired lactol as a pale yellow oil. The crude lactol was dissolved in CH_2Cl_2 (10 mL), and trichloroacetonitrile (5 mL) and DBU (10 μL) were added. The mixture was stirred for 1 h at room temperature and concentrated. The resulting residue was purified by flash chromatography (2:1 hexanes/EtOAc, 2% Et_3N) to yield 794 mg (76%) of compound **21** as a colorless oil. $[\alpha]_D$: +67.9 (c = 1.90, CH_2Cl_2). IR (thin film): 3337, 2920, 2871, 1739, 1718, 1672, 1353, 1289, 1155, 1103, 1074, 1027 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.57 (s, 1 H), 7.36−7.28 (m, 15 H), 6.54 (d, J = 3.5 Hz, 1 H), 5.39 (dd, J = 10.5, 2.9 Hz, 1 H), 4.76 (d, J = 11.3 Hz, 1 H), 4.73 (d, J = 10.3 Hz, 1 H), 4.64 (d, J = 12.1 Hz, 1 H), 4.57 (d, J = 11.4 Hz, 1 H), 4.50 (d, J = 11.8 Hz, 1 H), 4.43 (d, J = 11.8 Hz, 1 H), 4.30 (app t, J = 6.6 Hz, 1 H), 4.24 (dd, J = 10.5, 3.5 Hz, 1 H), 4.17 (app d, J = 2.3 Hz, 1 H), 3.59−3.56 (m, 2 H), 2.81−2.75 (m, 1 H), 2.66−2.40 (m, 3 H), 2.17 (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.80, 172.66, 161.71, 138.58, 138.36, 138.11, 128.83, 128.76, 128.67, 128.63, 128.54, 128.39, 128.24, 128.19, 128.15, 128.12, 127.95, 95.02, 91.63, 75.68, 75.32, 73.77, 73.62, 73.18, 72.80, 71.85, 68.21, 60.83, 38.18, 30.25, 28.32. ESI-MS: m/z (M + Na)$^+$ calcd 714.1399, obsd 714.1370.

Dibutyl 2,4,6-Tri-*O*-benzyl-3-*O*-levulinoyl-β-D-galactopyranoside Phosphate 4. Glycosyl trichloroacetimidate **21** (425 mg, 0.613 mmol) was dissolved in CH_2Cl_2 (5 mL) and cooled to 0 °C. Dibutyl phosphate (135 μL, 0.675 mmol) was added and the mixture was stirred for 1 h and concentrated. The residue was purified by flash chromatography (1:1 hexane/EtOAc, 2% Et_3N) to afford 410 mg (90%) of **4** as a colorless oil. $[\alpha]_D$: +28.8 (c = 0.92, CH_2Cl_2). IR (thin film): 2927, 1717, 1494, 1262, 1050 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36−7.27 (m, 15 H), 5.18 (app t, J = 7.0 Hz, 1 H), 4.96 (dd, J = 10.2, 3.1 Hz, 1 H), 4.86 (d, J = 11.6 Hz, 1 H), 4.72 (d, J = 11.6 Hz, 1 H), 4.67 (d, J = 11.6 Hz, 1 H), 4.56 (d, J = 11.6 Hz, 1 H), 4.47 (d, J = 11.8 Hz, 1 H), 4.42 (d, J = 11.8 Hz, 1 H), 4.10−3.98 (m, 5 H), 3.91 (dd, J = 10.2, 7.8 Hz, 1 H), 3.84−3.80 (m, 1 H), 3.61−3.59 (m, 2 H), 2.70−2.32 (m, 4 H), 2.13 (s, 3 H), 1.63−1.56 (m, 4 H) 1.38−1.24 (m, 4 H), 0.89 (t, J = 7.4 Hz, 3 H) 0.86 (t, J = 7.4 Hz, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.38, 172.25, 138.38, 138.29, 137.78, 128.58, 128.44, 128.42, 128.17, 127.95, 127.84, 127.83, 127.77, 99.13, 77.00, 75.21, 75.16, 74.88, 74.22, 73.85, 73.55, 67.87, 67.77, 37.86, 32.29, 32.25, 29.93, 27.92, 18.72, 13.75, 13.72. ^{31}P NMR (120 MHz, $CDCl_3$): δ −1.66. ESI-MS: m/z (M + Na)$^+$ calcd 763.3218, obsd 763.3227.

***n*-Pentenyl 2,4,6-Tri-*O*-benzyl-3-*O*-levulinoyl-α-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*O*-pivaloyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*O*-pivaloyl-β-D-glucopyranoside 22. Procedure A.** Disaccharide **16** (980 mg, 0.945 mmol) and glycosyl phosphate **4** (2.39 g, 3.22 mmol) were coevaporated three times with toluene, dissolved in CH_2Cl_2/Et_2O (1:4, 50 mL), and cooled to −78 °C. TBSOTf (740 μL, 3.2 mmol) was added and the mixture was stirred for 2 h while warming to −20 °C. Triethylamine (5 mL) was added and the mixture was concentrated under reduced pressure. The residue was purified by flash chromatography (4:1 → 3:1 hexane/EtOAc) to afford 1.10 g (79%) of **22** as a colorless oil.

Procedure B. Disaccharide **16** (78 mg, 0.083 mmol) and trichloroacetimidate **21** (105 mg, 0.166 mmol) were coevaporated three times with toluene, dissolved in CH_2Cl_2 (4 mL), and cooled to 0 °C. TMSOTf (5 μL, 0.025 mmol) was added and the mixture was stirred for 40 min at 0 °C. Triethylamine (50 μL) was added and the mixture was concentrated under reduced pressure. The residue was purified by flash chromatography (4:1 → 3:1 hexane/EtOAc) to afford 66 mg (54%) of **22** as a colorless oil.

$[\alpha]_D$: +23.7 (c = 0.91, CH_2Cl_2). IR (thin film): 2930, 2870, 1740, 1132, 1095, 1054 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ

7.40−7.16 (m, 35 H), 5.88−5.81 (m, 1 H), 5.40−5.35 (m, 2 H), 5.16 (d, J = 12.1 Hz, 1 H), 5.08−5.00 (m, 4 H), 4.81 (d, J = 12.1 Hz, 1 H), 4.80 (d, J = 12.1 Hz, 1 H), 4.65−4.51 (m, 8 H), 4.41−4.33 (m, 3 H), 4.23−4.22 (m, 2 H), 4.15−4.16 (m, 1 H), 4.13−4.06 (m, 5 H), 3.92−3.81 (m, 3 H), 3.71−3.67 (m, 1 H), 3.52−3.41 (m, 5 H), 3.32 (dd, J = 10.4, 2.4 Hz, 1 H), 3.12 (dd, J = 8.9, 4.9 Hz, 1 H), 2.57−2.54 (m, 1 H), 2.49−2.31 (m, 3 H), 2.17−2.14 (m, 2 H), 2.11 (s, 3 H), 1.72−1.69 (m, 2 H), 1.22 (s, 9 H), 1.18 (s, 9 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.64, 176.88, 176.54, 171.64, 139.56, 138.98, 138.70, 138.48, 138.41, 138.37, 138.33, 138.01, 128.60, 128.57, 128.53, 128.44, 128.31, 128.30, 128.22, 128.08, 127.99, 127.97, 127.89, 127.84, 127.79, 127.73, 127.71, 127.47, 127.45, 126.90, 114.96, 104.41, 101.45, 100.79, 81.29, 80.27, 76.35, 75.88, 75.41, 75.29, 75.13, 74.88, 74.65, 73.68, 73.63, 73.41, 73.24, 73.12, 72.62, 71.87, 71.31, 69.08, 68.66, 68.36, 67.70, 67.49, 38.93, 38.88, 37.97, 30.27, 29.99, 28.98, 28.00, 27.56, 27.31. ESI-MS: m/z (M + Na)$^+$ calcd 1491.7013, obsd 1491.7052.

***n*-Pentenyl 2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranoside 23.** To a deep blue solution of sodium in liquid ammonia (ca. 7 mL) was added trisaccharide **22** (125 mg, 0.085 mmol) in dry THF (5 mL) under N_2 at −78 °C. After 45 min the reaction was quenched with MeOH (5 mL) and most of the ammonia was removed with a stream of N_2. The mixture was diluted with MeOH, treated with Dowex 50-X8 ion-exchange resin (washed and dried), filtered, and rinsed thoroughly with MeOH. The solution was concentrated in vacuo, and the resulting residue was dissolved in pyridine (3 mL) and treated with Ac_2O (2 mL) in the presence of DMAP (one crystal) at room temperature for 18 h. Flash chromatography of the crude material (1:1 → 1:2 hexanes/EtOAc) afforded 36 mg (43%) of **23** as a colorless oil. $[\alpha]_D$: +44.9 (c = 1.20, CH_2Cl_2). IR (thin film): 2926, 1750, 1700, 1653, 1558, 1540, 1495, 1373, 1230, 1050 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.80−5.76 (m, 1 H), 5.60 (d, J = 1.8 Hz, 1 H), 5.40 (dd, J = 11.0, 2.9 Hz, 1 H), 5.24−5.17 (m, 2 H), 5.11 (dd, J = 10.5, 8.1 Hz, 1 H), 5.04−4.96 (m, 3 H), 4.90 (m, 1 H), 4.73 (app d, J = 10.8 Hz, 1 H), 4.54−4.27 (m, 4 H), 4.18−4.09 (m, 4 H), 4.02 (app s, 1 H), 3.87−3.77 (m, 4 H), 3.78−3.62 (m, 1 H), 3.52−3.46 (m, 1 H) 2.14 (s, 3 H), 2.13 (s, 3 H), 2.09−2.06 (m, 23 H), 2.13 (s, 3 H), 1.71−1.62 (m, 2 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.69, 170.51, 170.47, 170.10, 169.72, 169.69, 169.54, 168.87, 137.81, 115.07, 101.10, 100.53, 99.63, 73.14, 72.81, 72.45, 71.79, 71.74, 70.21, 69.30, 68.93, 68.82, 67.85, 67.09, 67.04, 62.23, 61.26, 60.39, 60.23, 53.44, 29.82, 28.56, 20.95, 20.88, 20.72, 20.69, 20.65, 20.61, 20.52. ESI-MS: m/z (M + Na)$^+$ calcd 1015.3265, obsd 1015.3253.

3-*O*-Acetyl-2-azido-4,6-di-*O*-benzyl-2-deoxy-α/*β*-D-galactopyranosyl Nitrate 25. Galactal **24**[40] (3.03 g, 8.22 mmol) was dissolved in CH_3CN (60 mL) and cooled to −15 °C. CAN (13.5 g, 24.7 mmol) and NaN_3 (800 mg, 12.3 mmol) were added, and the mixture was stirred vigorously using a mechanical stirrer. After 4 h the reaction mixture was diluted with ice-cold Et_2O, washed twice with ice−water, dried over Na_2SO_4, and concentrated. The crude residue was purified by flash chromatography (5:1 hexanes/EtOAc) to afford 1.7 g (45%) of **25** (α:*β* = 4:1) as a colorless oil. IR (thin film): 2922, 2116, 1748, 1652, 1280, 1223, 1102, 1028 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, selected peaks): δ 6.30 (d, J = 4.1 Hz, 1 H, α H-1), 5.54 (d, J = 8.8 Hz, 1 H, *β* H-1), 5.19 (dd, J = 11.3, 2.9 Hz, 1 H, α H-3), 4.86 (dd, J = 11.0, 3.0 Hz, 1 H, *β* H-3), 4.29 (dd, J = 11.3, 4.2 Hz, 1 H, α H-2), 4.18 (app d, J = 2.9 Hz, 1 H, α H-4), 4.07 (app d, J = 2.9 Hz, 1 H, *β* H-4), 4.00 (dd, J = 11.0, 8.8 Hz, 1 H, *β* H-2). ESI-MS: m/z (M + Na)$^+$ calcd 495.1486, obsd 495.1469.

3-*O*-Acetyl-2-azido-4,6-di-*O*-benzyl-2-deoxy-D-galactose 26. To a solution of **25** (1.42 g, 3.00 mmol) in CH_3CN (30 mL) were added thiophenol (900 μL, 9.00 mmol) and DIEA (525 μL, 3.00 mmol) at 0 °C. After 90 min the reaction mixture was concentrated under reduced pressure and the crude residue purified by flash chromatography (6:1 → 2:1 hexanes/EtOAc) to give 1.16 g (91%, α:*β* = 2:1) of **26** as a white solid. IR (thin film): 3399, 3031, 2872, 2112, 1745, 1231, 1045 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$, selected peaks): δ 5.38 (d, J = 3.4 Hz, 1 H, α H-1), 5.32 (dd, J = 11.0, 2.9 Hz, 1 H, α H-3), 4.74 (dd, J = 10.8, 3.1 Hz, 1 H, *β* H-3), 4.06 (app d, J = 2.3 Hz, α H-4), 3.94 (app d, J = 3.3 Hz, 1 H, *β* H-4), 3.92 (dd, J = 11.1, 3.5 Hz, α H-2), 3.07 (br s, 1 H, OH). ESI-MS: m/z (M + Na)$^+$ calcd 450.1636, obsd 450.1628.

Dimethylthexylsilyl 3-*O*-Acetyl-2-azido-4,6-di-*O*-benzyl-2-deoxy-*β*-D-galactopyranoside 27. To 1.16 g (2.70 mmol) of **26** in DMF (25 mL) were added imidazole (551 mg, 8.10 mmol) and thexyldimethylsilyl chloride (TDS−Cl) (800 μL, 4.07 mmol). The mixture was stirred at room temperature for 12 h, diluted with EtOAc, and washed with water, saturated aqueous $NaHCO_3$, water, and brine. The organic phase was dried over $MgSO_4$, concentrated, and purified by flash chromatography (8:1 hexanes/EtOAc) to afford 1.48 g (96%) of **27** as a colorless oil. $[\alpha]_D$: +2.4 (c = 1.32, CH_2Cl_2). IR (thin film): 2927, 2112, 1749, 1653, 1558, 1455, 1259, 1090 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.49−7.22 (m, 10 H), 4.70 (dd, J = 11.1, 2.7 Hz, 1 H), 4.63−4.40 (m, 5 H), 3.94 (app s, 1 H), 3.74 (dd, J = 8.0, 2.4 Hz, 1 H), 3.66−3.59 (m, 3 H), 2.04 (s, 3 H), 1.69 (m, 1 H), 0.90 (m, 12 H), 0.21 (s, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.25, 138.08, 137.95, 137.74, 137.70, 129.34, 128.61, 128.46, 128.43, 128.38, 128.18, 128.15, 128.07, 127.86, 127.79, 127.74, 126.05, 97.31, 75.30, 75.24, 75.05, 73.58, 73.53, 73.47, 73.33, 72.41, 68.48, 68.23, 67.38, 63.83, 33.80, 24.81, 20.87, 19.95, 19.83, 18.50, 18.40, 1.03, −1.91, −3.25, −3.27. ESI-MS: m/z (M + Na)$^+$ calcd 592.2813, obsd 592.2805.

Dimethylthexylsilyl 3-*O*-Acetyl-4,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-*β*-D-galactopyranoside 28. To a solution of **27** (1.34 g, 2.35 mmol) in EtOH (20 mL) were added $NaBH_4$ (444 mg, 11.8 mmol) and $NiCl_2$ (56 mg, 0.24 mmol). The solution was stirred for 90 min at room temperature then neutralized with AcOH and concentrated to dryness. The residue was precipitated with CH_2Cl_2, filtered over a pad of Celite, and concentrated. To a solution of the crude amine in CH_2Cl_2 (20 mL) was added Et_3N (975 μL, 7.00 mmol) and trichloroacetyl chloride (310 μL, 2.80 mmol) at 0 °C. The reaction mixture was stirred for 20 min at 0 °C and then diluted with CH_2Cl_2 and washed with water, saturated aqueous $NaHCO_3$, and water. The organic phase was dried over $MgSO_4$ and concentrated. The resulting residue was dissolved in pyridine (10 mL) and treated with Ac_2O (5 mL). After 12 h the reaction mixture was concentrated, coevaporated with toluene, and purified by flash chromatography (4:1 hexanes/EtOAc) to yield 648 mg (40%) of **28** as a colorless oil. $[\alpha]_D$: −5.3 (c = 0.89, CH_2Cl_2). IR (thin film): 2926, 2862, 1717, 1653, 1558, 1540, 1403, 1050 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36−7.27 (m, 10 H), 6.67 (d, J = 9.0 Hz, 1 H), 5.19 (dd, J = 11.3, 2.3 Hz, 1 H), 4.85 (d, J = 7.8 Hz, 1 H), 4.75 (d, J = 11.7 Hz, 1 H), 4.60 (d, J = 11.7 Hz, 1 H), 4.53 (d, J = 11.7 Hz, 1 H), 4.47 (d, J = 11.7 Hz, 1 H), 4.31−4.24 (m, 1 H), 3.98 (app s, 1 H), 3.77−3.74 (m, 1 H), 3.68 (app t, J = 8.8 Hz, 1 H), 3.64−3.62 (m, 1 H), 2.00 (s, 3 H), 1.65−1.62 (m, 1 H), 0.88−0.85 (m, 12 H), 0.20 (s, 3 H), 0.16 (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.34, 161.98, 138.38, 138.19, 129.44, 128.87, 128.78, 128.68, 128.25, 128.19, 96.52, 93.02, 75.37, 74.06, 73.98, 73.92, 68.73, 55.86, 34.23, 25.16, 21.22, 20.45, 20.36, 18.98, 18.96, −1.22, −2.86. ESI-MS: m/z (M + Na)$^+$ calcd 710.1845, obsd 710.1867.

3-*O*-Acetyl-4,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-α-D-galactopyranosyl Trichloroacetimidate 30. To a solution of compound **28** (740 mg, 1.07 mmol) in dry THF (10 mL) were added AcOH (75 μL, 1.3 mmol) and a 1.0 M solution of tetrabutylammonium fluoride in THF (1.3 mL, 1.3 mmol). The mixture was stirred at room temperature for 20 h, diluted with EtOAc, washed with saturated aqueous $NaHCO_3$, water, and brine, dried over $MgSO_4$, and concentrated. Purification of the resulting residue by flash chromatography (8:1 → 4:1 hexanes/EtOAc) yielded 530 mg (91%) of **29** as a colorless oil. To a solution of lactol **29** (527 mg, 0.96

mmol) in CH_2Cl_2 (10 mL) was added trichloroacetonitrile (5 mL) and DBU (10 μL). The mixture was stirred at room temperature for 2 h and then concentrated. Purification of the crude residue by flash chromatography (4:1 hexanes/EtOAc) yielded 461 mg (70%) of **30** as a colorless foam. $[\alpha]_D$: +65.1 (c = 0.83, CH_2Cl_2). IR (thin film): 2926, 1717, 1684, 1558, 1494, 1452, 1403, 1050 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.73 (s, 1 H), 7.39−7.27 (m, 10 H), 6.93 (d, J = 8.9 Hz, 1 H), 6.47 (d, J = 3.5 Hz, 1 H), 5.42 (dd, J = 11.3, 2.7 Hz, 1 H), 4.90−4.87 (m, 1 H), 4.82 (d, J = 11.4 Hz, 1 H), 4.62 (d, J = 11.4 Hz, 1 H), 4.52−4.49 (m, 2 H), 4.44 (d, J = 11.7 Hz, 1 H), 4.25 (dd, J = 7.8, 5.7 Hz, 1 H), 4.12 (d, J = 2.4 Hz, 1 H), 3.70 (app t, J = 9.0 Hz, 1 H), 3.61 (dd, J = 9.1, 5.4 Hz, 1 H), 2.06 (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.86, 162.44, 160.67, 138.06, 137.94, 128.89, 128.87, 128.58, 128.39, 128.34, 128.28, 95.32, 92.46, 91.22, 77.64, 75.65, 74.06, 73.92, 72.47, 70.95, 67.80, 50.92, 21.28. ESI-MS: m/z (M + Na)$^+$ calcd 710.9763, obsd 710.9763.

Dibutyl 3-*O*-Acetyl-4,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-*β*-D-galactopyranosyl Phosphate 5. To a solution of imidate **30** (453 mg, 0.66 mmol) in CH_2Cl_2 (5 mL) at 0 °C was added dibutyl phosphate (150 μL, 0.72 mmol). The mixture was stirred for 1 h and then directly loaded onto a short column of silica gel and eluted with 1:1 hexanes/EtOAc to afford 360 mg (74%) of phosphate **5** as a white solid. $[\alpha]_D$: +13.8 (c = 0.82, CH_2Cl_2). IR (thin film): 2928, 1718, 1540, 1494, 1454, 1260, 1028 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.08 (d, J = 9.5 Hz, 1 H), 7.35−7.26 (m, 10 H), 5.53 (app t, J = 7.8 Hz, 1 H), 5.25 (d, J = 10.9 Hz, 1 H), 4.71 (d, J = 11.6 Hz, 1 H), 4.63−4.55 (m, 1 H), 4.50 (d, J = 11.2 Hz, 1 H), 4.47 (d, J = 10.9 Hz, 1 H), 4.38 (d, J = 11.7 Hz, 1 H), 4.09 (m, 2 H), 4.02−3.99 (m, 2 H), 3.82 (app s, 2 H), 3.64−3.60 (m, 1 H), 3.53−3.49 (m, 1 H), 2.01 (s, 3 H), 1.64−1.59 (m, 4 H), 1.38−1.36 (m, 4 H), 0.94−0.88 (m, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.41, 163.73, 162.42, 137.60, 128.35, 128.29, 128.19, 127.82, 127.70, 127.53, 96.77, 96.72, 92.62, 91.97, 74.92, 73.31, 73.04, 72.81, 68.34, 68.27, 68.14, 68.10, 68.03, 67.66, 52.72, 52.63, 31.93, 31.86, 20.68, 18.49, 18.46, 13.49. ^{31}P NMR (120 MHz, $CDCl_3$): δ −2.62. ESI-MS: m/z (M + Na)$^+$ calcd 760.1582, obsd 760.1559.

***n*-Pentenyl 2,4,6-Tri-*O*-benzyl-α-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*O*-pivaloyl-*β*-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*O*-pivaloyl-*β*-D-glucopyranoside 31.** Trisaccharide **22** (1.10 g, 0.746 mmol) was dissolved in CH_2Cl_2 (20 mL). A solution of hydrazine acetate (122 mg, 1.33 mmol) in MeOH (4 mL) was added and the mixture was stirred for 12 h. The mixture was diluted with CH_2Cl_2 (100 mL) and washed twice with water, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (4:1 → 2:1 hexane/EtOAc) to afford 634 mg (62%) of **31** as a colorless oil. $[\alpha]_D$: +25.1 (c = 1.00, CH_2Cl_2). IR (thin film): 3030, 2871, 1737, 1130, 1094 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37−7.16 (m, 35 H), 5.83−5.67 (m, 1 H), 5.27 (dd, J = 10.4, 7.9 Hz, 1 H), 5.14 (d, J = 11.9 Hz, 1 H), 5.04−4.94 (m, 4 H), 4.78 (d, J = 12.2 Hz, 1 H), 4.77 (d, J = 12.1 Hz, 1 H), 4.66 (d, J = 11.3 Hz, 1 H), 4.56−4.45 (m, 6 H), 4.38−4.34 (m, 2 H), 4.24−4.20 (m, 3 H), 4.12−3.99 (m, 5 H), 3.96−3.95 (m, 1 H), 3.88−3.81 (m, 1 H), 3.73−3.79 (m, 3 H), 3.62 (app t, J = 9.0 Hz, 1 H), 3.50−3.34 (m, 5 H), 3.27 (dd, J = 10.4, 2.5 Hz, 1 H), 3.07 (dd, J = 8.8, 4.9 Hz, 1 H), 2.12−2.05 (m, 2 H), 1.78−1.62 (m, 3 H), 1.17 (s, 9 H), 1.12 (s, 9 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.90, 176.74, 139.44, 139.01, 138.62, 138.36, 138.32, 138.29, 138.24, 138.13, 128.61, 128.42, 128.39, 128.31, 128.28, 128.21, 128.17, 128.11, 127.92, 127.86, 127.75, 127.68, 127.60, 127.46, 127.30, 127.16, 114.96, 101.47, 100.19, 99.94, 81.35, 80.03, 75.40, 75.33, 75.30, 74.60, 74.48, 73.68, 73.51, 73.31, 73.12, 73.12, 72.36, 71.75, 71.30, 70.09, 69.08, 68.94, 68.30, 67.69, 67.55, 30.23, 28.93, 28.93, 27.25. ESI-MS: m/z (M + Na)$^+$ calcd 1393.6645, obsd 1393.6612.

***n*-Pentenyl 3-*O*-Acetyl-4,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-*β*-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*O*-pivaloyl-*β*-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*O*-pivaloyl-*β*-D-glucopyranoside 32.** Trisaccharide **31** (308 mg, 0.230 mmol) and glycosyl phosphate **5** (196 mg, 0.270 mmol) were coevaporated three times with toluene, dissolved in CH_2Cl_2 (4 mL), and cooled to −78 °C. TMSOTf (50 μL, 0.270 mmol) was added and the mixture was stirred for 30 min. Triethylamine (200 μL) was added and the mixture was directly purified by flash chromatography (4:1 → 2:1 hexane/EtOAc) to afford 405 mg (93%) of **32** as a white solid. $[\alpha]_D$: −15.2 (c = 1.00, CH_2Cl_2). IR (thin film): 1734, 1521, 1455, 1364, 1231, 1093 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.46−7.18 (m, 45 H), 6.54 (d, J = 9.8 Hz, 1 H), 5.85−5.78 (m, 1 H), 5.36 (dd, J = 9.9, 8.3 Hz, 1 H), 5.06−4.96 (m, 4 H), 4.84−4.74 (m, 6 H), 4.66−4.47 (m, 8 H), 4.38−4.02 (m, 17 H), 4.02−3.99 (m, 2 H), 3.89 (app s, 1 H), 3.90−3.80 (m, 2 H), 3.78−3.51 (m, 3 H), 3.48−3.35 (m, 4 H), 3.28 (app d, J = 10.2 Hz, 1 H), 3.05 (dd, J = 8.5, 5.1 Hz, 1 H), 2.12−2.08 (m, 2 H), 2.00 (s, 3 H), 1.71−1.66 (m, 2 H), 1.18 (s, 9 H), 1.26 (s, 9 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.27, 171.13, 163.83, 162.32, 139.08, 138.55, 138.50, 138.46, 138.43, 138.37, 129.72, 129.15, 129.04, 128.93, 128.89, 128.86, 128.83, 128.65, 128.63, 128.45, 128.40, 128.29, 128.25, 128.21, 128.15, 128.13, 128.03, 127.94, 127.92, 127.85, 127.80, 127.57, 127.42, 115.26, 102.34, 101.73, 101.00, 92.84, 80.41, 80.00, 79.64, 77.72, 77.64, 75.48, 75.39, 75.27, 75.15, 74.89, 74.24, 73.96, 73.93, 73.85, 73.80, 73.61, 73.50, 73.38, 73.04, 73.01, 72.33, 72.16, 71.84, 71.76, 69.34, 69.13, 68.48, 68.17, 67.93, 67.64, 53.65, 39.19, 39.09, 30.49, 29.21, 27.76, 27.67, 27.58, 21.32. ESI-MS: m/z (M + Na)$^+$ calcd 1920.7315, obsd 1920.7340.

***n*-Pentenyl 4,6-Di-*O*-benzyl-2-deoxy-2-trichloroacetamido-*β*-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*O*-pivaloyl-*β*-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*O*-pivaloyl-*β*-D-glucopyranoside 33.** To a solution of tetrasaccharide **32** (305 mg, 0.160 mmol) in MeOH (15 mL) was added a solution of NaOMe in MeOH (180 μL, 0.80 mmol, 25% by wt). The reaction mixture was stirred at room temperature for 1 h and then quenched with Dowex 50-X8 ion-exchange resin, filtered, and concentrated. The crude residue was purified by flash chromatography (3:1 hexanes/EtOAc) to afford 262 mg (88%) of tetrasaccharide **33** as a white solid. $[\alpha]_D$: −6.6 (c = 1.10, CH_2Cl_2). IR (thin film): 2870, 1735, 1454, 1366, 1094 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.45−7.18 (m, 45 H), 6.37 (d, J = 8.7 Hz, 1 H), 5.86−5.76 (m, 1 H), 5.37 (app t, J = 8.2 Hz, 1 H), 5.04−4.90 (m, 5 H), 4.80−4.65 (m, 6 H), 4.56−4.30 (m, 8 H), 4.24 (app s, 1 H), 4.16−4.00 (m, 11 H), 3.86−3.67 (m, 6 H), 3.66−3.57 (m, 2 H), 3.44−3.28 (m, 5 H), 3.17 (app d, J = 10.5 Hz, 1 H), 3.08 (dd, J = 8.3, 5.4 Hz, 1 H), 2.34 (br s, 1 H), 1.87−1.89 (m, 2 H), 1.88 (s, 9 H), 1.69−1.64 (m, 2 H), 1.13 (s, 9 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.84, 176.81, 139.44, 139.31, 138.79, 138.31, 138.24, 138.20, 138.15, 138.09, 129.44, 128.72, 128.66, 128.61, 128.58, 128.38, 128.24, 128.18, 128.09, 128.04, 127.97, 127.93, 127.86, 127.73, 127.66, 127.60, 127.38, 127.25, 114.98, 101.66, 101.43, 100.30, 100.07, 92.47, 79.83, 79.59, 79.20, 75.61, 75.51, 75.27, 75.14, 73.78, 73.71, 73.64, 73.48, 73.36, 73.17, 72.93, 72.81, 72.30, 71.64, 71.42, 69.03, 68.26, 67.87, 67.85, 67.42, 56.52, 38.93, 38.83, 30.22, 28.94, 28.30, 27.52, 27.32, 22.89, 14.43. ESI-MS: m/z (M + Na)$^+$ calcd 1878.7209, obsd 1878.7262.

Dibutyl 2-*O*-Acetyl-3,4,6-tri-*O*-benzyl-*β*-D-galactopyranosyl Phosphate 6. Tri-*O*-benzyl galactal **12** (644 mg, 1.55 mmol) was dissolved in CH_2Cl_2 (15 mL) and cooled to 0 °C. DMDO (0.08 M in acetone, 30 mL, 2.4 mmol) was added and the reaction was stirred for 10 min. The solvent was evaporated at 0 °C, and the resulting residue was dissolved in CH_2Cl_2 (15 mL) and cooled to −78 °C. Dibutyl phosphate (340 μL, 1.7 mmol) was added and the mixture was stirred for 10 min. After warming to 0 °C DMAP (758 mg, 6.20 mmol) and acetyl chloride (220 μL, 3.10 mmol) were added, and the reaction mixture was stirred for 2 h and concentrated. Purification of the crude residue by flash chromatography (1:1

hexane/EtOAc) afforded 769 mg (73%) of phosphate **6** as a colorless oil. $[\alpha]_D$: +25.9 (c = 0.93, CH_2Cl_2). IR (thin film): 2927, 1751, 1558, 1494, 1452, 1403, 1050 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 7.54−7.28 (m, 15 H), 5.46 (dd, J = 10.0, 8.0 Hz, 1 H), 5.16 (app t, J = 7.5 Hz, 1 H), 4.96 (d, J = 11.5 Hz, 1 H), 4.69 (d, J = 12.4 Hz, 1 H), 4.62 (d, J = 11.4 Hz, 1 H), 4.53 (d, J = 12.2 Hz, 1 H), 4.45 (app s, 2 H), 4.07−3.95 (m, 5 H), 3.71−3.65 (m, 2 H), 3.62−3.55 (m, 2 H), 2.05 (s, 3 H), 1.62−1.40 (m, 4 H), 1.38−1.35 (m, 4 H), 0.95−0.87 (m, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.48, 138.24, 137.61, 137.59, 128.44, 128.22, 127.85, 127.64, 127.51, 127.37, 96.97, 79.64, 74.63, 74.13, 73.49, 72.20, 72.08, 67.94, 67.87, 67.81, 32.08, 32.01, 31.94, 20.92, 18.55, 13.55, 13.53. ^{31}P NMR (120 MHz, $CDCl_3$): δ −2.26. ESI-MS: m/z (M + Na)$^+$ calcd 707.2956, obsd 707.2931.

***n*-Pentenyl 2-*O*-Acetyl-3,4,6-tri-*O*-benzyl-*β*-D-galactopyranosyl-(1→3)-4,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-*β*-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*O*-pivaloyl-*β*-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*O*-pivaloyl-*β*-D-glucopyranoside 36.** Tetrasaccharide **33** (89 mg, 48 μmol) and glycosyl phosphate **6** (66 mg, 96 μmol) were coevaporated three times with toluene, dissolved in CH_2Cl_2 (4 mL), and cooled to −50 °C. TMSOTf (18 μL, 96 μmol) was added and the mixture was stirred for 20 min. Triethylamine (100 μL) was added and the mixture was directly purified by flash chromatography (4:1 hexanes/EtOAc) to afford 99 mg (88%) of pentasaccharide **36** as a colorless foam. $[\alpha]_D$: +1.8 (c = 1.00, CH_2Cl_2). IR (thin film): 2926, 1734, 1700, 1653, 1558, 1495, 1455, 1400, 1050 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 7.35−7.18 (m, 60 H), 6.22 (d, J = 8.9 Hz, 1 H), 5.84−5.77 (m, 1 H), 5.45 (app t, J = 8.6 Hz, 1 H), 5.32 (app t, J = 8.8 Hz, 1 H), 5.04−4.96 (m, 6 H), 4.86−3.53 (m, 48 H), 3.43−3.36 (m, 5 H), 3.26−3.24 (m, 2 H), 3.15−3.13 (m, 1 H), 2.10−2.06 (m, 2 H), 2.06 (s, 3 H), 1.70−1.65 (m, 2 H), 1.15 (s, 9 H), 1.12 (s, 9 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.55, 176.37, 169.76, 160.94, 139.34, 139.18, 139.02, 138.63, 138.57, 138.45, 138.40, 138.06, 137.87, 137.74, 137.65, 128.74, 128.52, 128.49, 128.37, 128.34, 128.31, 128.26, 128.21, 128.13, 128.07, 127.99, 127.91, 127.88, 127.84, 127.81, 127.73, 127.66, 127.58, 127.52, 127.41, 127.35, 127.11, 127.02, 126.90, 114.75, 101.24, 101.15, 100.76, 100.10, 92.68, 79.64, 78.06, 77.00, 76.49, 75.88, 75.07, 74.81, 74.77, 74.49, 73.87, 73.58, 73.46, 73.24, 73.16, 72.96, 72.72, 72.33, 71.84, 71.43, 71.10, 70.79, 68.94, 68.80, 68.46, 68.40, 67.60, 55.20, 38.67, 38.60, 30.02, 29.68, 29.26, 28.75, 28.10, 27.30, 27.10, 22.43, 21.10, 13.98. ESI-MS: m/z (M + Na)$^+$ calcd 2352.9251, obsd 2352.9288.

***n*-Pentenyl 3,4,6-Tri-*O*-benzyl-*β*-D-galactopyranosyl-(1→3)-4,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-*β*-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*O*-pivaloyl-*β*-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*O*-pivaloyl-*β*-D-glucopyranoside 37.** To a solution of pentasaccharide **36** (98 mg, 42 μmol) in MeOH (3 mL) was added a solution of NaOMe in MeOH (48 μL, 21 μmol, 25% by wt). The reaction mixture was stirred at room temperature for 12 h and then quenched with Dowex 50-X8 ion-exchange resin, filtered, and concentrated. The crude residue was purified by flash chromatography (4:1 hexanes/EtOAc) to afford 73 mg (76%) of pentasaccharide **37** as a colorless foam. $[\alpha]_D$: −0.3 (c = 1.00, CH_2Cl_2). IR (thin film): 2926, 1734, 1558, 1494, 1403, 1261, 1050 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 7.38−7.16 (m, 60 H), 6.51 (d, J = 8.5 Hz, 1 H), 5.84−5.74 (m, 1 H), 5.32 (app t, J = 8.4 Hz, 1 H), 4.99−4.88 (m, 7 H), 4.76−4.25 (m, 22 H), 4.18−3.74 (m, 17 H), 3.60−3.54 (m, 8 H), 3.41−3.34 (m, 5 H), 3.26−3.24 (m, 2 H), 3.10 (dd, J = 8.6, 5.2 Hz, 1 H), 2.44 (br s, 1 H), 2.09−2.06 (m, 2 H), 1.67−1.65 (m, 2 H), 1.16 (s, 9 H), 1.12 (s, 9 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.55, 176.42, 162.11, 139.26, 139.16, 139.01, 138.66, 138.61, 138.38, 138.38, 138.26, 138.05, 138.03, 137.88, 137.73, 128.97, 128.55, 128.47, 128.42, 128.39, 128.34, 128.24, 128.15, 128.07, 128.00, 127.93, 127.91, 127.83, 127.76, 127.67, 127.61, 127.52, 127.40, 127.35, 127.10, 127.05, 127.01, 114.75, 104.39, 101.17, 100.07, 99.99, 92.57, 80.99, 79.80, 79.68, 79.56, 78.25, 75.81, 75.55, 75.06, 74.88, 74.59, 74.47, 73.35, 73.92, 73.87, 73.57, 73.46, 73.34, 73.28, 73.22, 72.93, 72.85, 72.71, 72.39, 72.26, 71.70, 71.40, 71.15, 68.91, 68.79, 68.72, 68.25, 68.15, 67.82, 67.47, 38.68, 38.59, 31.90, 31.56, 30.02, 29.67, 29.34, 29.25, 28.74, 28.10, 27.30, 27.10, 22.63, 22.42, 21.05, 14.17, 14.12, 13.97. ESI-MS: m/z (M + Na)$^+$ calcd 2310.9146, obsd 2310.9136.

***n*-Pentenyl 2,3,4,6-Tetra-*O*-acetyl-*β*-D-galactopyranosyl-(1→3)-4,6-di-*O*-acetyl-2-deoxy-2-acetamido-*β*-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-acetyl-α-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranoside 38.** Pentasaccharide **37** (35 mg, 15 μmol), Bu_3SnH (50 μL, 160 μmol), and a catalytic amount of AIBN were dissolved in dry toluene (3 mL), and the solution was vigorously stirred for 20 min under a stream of N_2. After heating to 100 °C for 1 h, another 20 μL of Bu_3SnH and a catalytic amount of AIBN were added. After 1 h the reaction mixture was cooled to room temperature and concentrated under reduced pressure. The residue was purified by flash chromatography (10:1 → 4:1 → 2:1 hexanes/EtOAc) to afford 18 mg (55%) of the desired product. To a deep blue solution of sodium in liquid ammonia (ca. 7 mL) was added the above compound in dry THF (3 mL) under N_2 at −78 °C. After 45 min, the reaction was quenched with MeOH (4 mL) and most of the ammonia was removed with a stream of N_2. The mixture was diluted with MeOH, treated with Dowex 50-X8 ion-exchange resin (washed and dried), filtered, and rinsed with a solution of NH_3 in MeOH. The solution was concentrated in vacuo and coevaporated with toluene. The resulting residue was dissolved in pyridine (2 mL) and treated with Ac_2O (1 mL) in the presence of DMAP (one crystal) at room temperature for 20 h. Flash chromatography of the crude material (2:1 EtOAc/hexanes → 100% EtOAc) gave 5 mg (40%) of pentasaccharide **38** as a white solid. IR (thin film): 1745, 1548, 1370, 1229, 1066 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 5.85−5.75 (m, 1 H), 5.67 (d, J = 7.2 Hz, 1 H), 5.61 (app s, 1 H), 5.42 (app s, 1 H), 5.36 (app d, J = 2.1 Hz, 1 H), 5.32−4.89 (m, 10 H), 4.77 (app d, J = 9.4 Hz, 1 H), 4.70 (app d, J = 8.4 Hz, 1 H), 4.63 (app d, J = 7.7 Hz, 1 H), 4.55 (d, J = 7.6 Hz, 1 H), 4.48 (app d, J = 8.0 Hz, 1 H), 4.44−4.37 (m, 2 H), 4.24−4.02 (m, 9 H), 3.96−3.93 (m, 1 H), 3.89−3.86 (m, 2 H), 3.81−3.77 (m, 2 H), 3.65−3.62 (m, 2 H), 3.52−3.48 (m, 1 H), 3.28−3.23 (m, 1 H), 2.16−1.96 (m, 48 H), 1.75−1.65 (m, 2 H), 1.61 (s, 3 H). ESI-MS: m/z (M + Na)$^+$ calcd 1590.5115, obsd 1590.5170.

***n*-Pentenyl 2-*O*-Benzyl-3,4-di-*O*-pivaloyl-α-L-fucopyranosyl-(1→2)-3,4,6-tri-*O*-benzyl-*β*-D-galactopyranosyl-(1→3)-4,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-*β*-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-benzyl-α-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*O*-pivaloyl-*β*-D-galactopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*O*-pivaloyl-*β*-D-glucopyranoside 1.** Pentasaccharide **37** (54 mg, 24 μmol) and fucosyl phosphate **7** (44 mg, 71 μmol) were coevaporated three times with toluene, dissolved in CH_2Cl_2 (2 mL), and cooled to −50 °C. TMSOTf (13 μL, 71 μmol) was added and the mixture was stirred for 45 min while warming to −20 °C. Triethylamine (200 μL) was added and the mixture was directly purified by flash chromatography (8:1 → 5:1 hexanes/EtOAc) to afford 42 mg (66%) of hexasaccharide **1** as a colorless foam. $[\alpha]_D$: −20.6 (c = 1.00, CH_2Cl_2). IR (thin film): 2926, 1734, 1558, 1494, 1403, 1262 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 7.56 (d, J = 6.7 Hz, 1 H), 7.38−7.15 (m, 60 H), 7.03−7.00 (m, 5 H), 5.86−5.72 (m, 1 H), 5.65 (d, J = 2.6 Hz, 1 H), 5.51 (app d, J = 10.6 Hz, 1 H), 5.30 (app t, J = 9.1 Hz, 1 H), 5.22 (app s, 1 H), 5.17−5.13 (m, 2 H), 5.07−4.86 (m, 7 H), 4.78−3.92 (m, 38 H), 3.92−3.58 (m, 10 H), 3.47−3.38 (m, 7 H), 3.24−3.18 (m, 2 H), 2.09−2.07 (m, 2 H), 1.67−1.64 (m, 2 H), 1.60 (s, 9 H), 1.15 (s, 9 H), 1.11 (s, 9 H), 1.04 (s, 9 H), 0.70 (d, J = 6.2 Hz, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.46, 177.26, 177.03, 176.69, 162.79, 140.06, 139.30, 139.18, 139.04, 138.78, 138.63, 138.56, 138.37, 138.24, 138.21, 128.95, 128.93, 128.86, 128.78, 128.75, 128.71, 128.62,

128.58, 128.55, 128.49, 128.40, 128.35, 128.27, 128.24, 128.07, 128.02, 127.97, 127.91, 127.84, 127.76, 127.69, 127.52, 127.45, 127.41, 127.34, 127.19, 115.19, 102.74, 101.52, 101.16, 100.91, 100.06, 96.93, 84.12, 80.68, 78.51, 78.16, 77.65, 76.66, 76.51, 75.69, 75.56, 75.44, 74.96, 74.85, 74.72, 74.52, 74.44, 74.00, 73.88, 73.72, 73.37, 73.11, 73.05, 72.39, 72.15, 71.94, 71.81, 71.75, 70.54, 69.25, 69.09, 68.86, 68.59, 68.31, 65.67, 39.30, 39.14, 39.08, 32.02, 30.50, 29.22, 27.73, 27.63, 27.57, 27.51, 23.09, 15.74, 14.57. ESI-MS: *m/z* $(M + 2Na)^{2+}$ calcd 1369.0618, obsd 1369.0602.

***n*-Pentenyl 2,3,4-Tri-*O*-acetyl-α-L-fucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→3)-2-acetamido-4,6-di-*O*-acetyl-2-deoxy-β-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-acetyl-α-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside 39.**[33] Hexasaccharide **1** (21 mg, 7.8 μmol), Bu_3SnH (25 μL, 80 μmol), and a catalytic amount of AIBN were dissolved in dry toluene (3 mL), and the solution was vigorously stirred for 20 min under a stream of N_2. After heating to 100 °C for 1 h, another 25 μL of Bu_3SnH and a catalytic amount of AIBN were added. After 1.5 h the reaction mixture was cooled to room temperature and concentrated under reduced pressure. The residue was purified by flash chromatography (10:1 → 4:1 → 2:1 hexanes/EtOAc) to afford 12 mg (60%) of the desired product. To a deep blue solution of sodium in liquid ammonia (ca. 7 mL) was added the above compound in dry THF (3 mL) under N_2 at −78 °C. After 45 min the reaction was quenched with MeOH (4 mL) and most of the ammonia was removed with a stream of N_2. The mixture was diluted with MeOH, treated with Dowex 50-X8 ion-exchange resin (washed and dried), filtered, and rinsed with a solution of NH_3 in MeOH. The solution was concentrated in vacuo and coevaporated with toluene. The resulting residue was dissolved in pyridine (2 mL) and treated with Ac_2O (1 mL) in the presence of DMAP (one crystal) at room temperature for 16 h. Flash chromatography of the crude material (2:1 EtOAc/hexanes → 100% EtOAc) afforded 4 mg (40%) of hexasaccharide **39** as a white solid. ^1H NMR (400 MHz, $CDCl_3$): δ 6.69 (d, J = 6.8 Hz, 1 H), 5.82−5.75 (m, 1 H), 5.61 (app s, 1 H), 5.48 (app d, J = 3.3 Hz, 1 H), 5.41 (app d, J = 2.4 Hz, 1 H), 5.31−4.85 (m, 12 H), 4.78−4.74 (m, 1 H), 4.54−4.40 (m, 6 H), 4.37−4.35 (m, 1 H), 4.26 (dd, J = 10.5, 2.5 Hz, 1 H), 4.21−3.96 (m, 8 H), 3.90−3.73 (m, 5 H), 3.64−3.60 (m, 1 H), 3.52−3.46 (m, 1 H), 3.08−3.04 (m, 1 H), 2.16−1.90 (m, 53 H), 1.68−1.64 (m, 2 H), 1.60 (s, 3 H), 1.15 (d, J = 6.2 Hz, 3 H). ESI-MS: *m/z* $(M + Na)^+$ calcd 1820.5906, obsd 1820.5949.[33]

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Supporting Information Available: ^1H NMR and ^{13}C NMR spectral data for all described compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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